9-5-00

A

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No. A-67689-3/RFT/RMS/RMK

"EXPRESS MAIL" MAILING LABEL NUMBER EK996771484US
DATE OF DEPOSIT September 1, 2000
I HEREBY CERTIFY THAT THIS PAPER OR FEE IS BEING DEPOSITE WITH THE UNITED STATES POSTAL SERVICE "EXPRESS MAIL POS OFFICE TO ADDRESSEE" SERVICE UNDER 37 C.F.R. 1.10 ON TH DATE INDICATED ABOVE AND IS ADDRESSED TO: ASSISTAN COMMISSIONER FOR PATENTS, BOX PATENT APPLICATION NO FEE WASHINGTON, DC 20231.
TYPED NAME VINCE DIAZ
SIGNED Vincent M Diaz

Anticipated Classification of this Application:

Class: Subclass:

This Application

Examiner:

Art Unit:

jc588 U.S. PTO 09/01/00

jc564 U.S. PTO 09/653924 09/01/00

Assistant Commissioner for Patents
BOX PATENT APPLICATION NO FEE
Washington, DC 20231

Sir:

This is a request for filing an

X Continuation-in-part

application under 37 C.F.R. 1.53(b), in the name of

David A. HORWITZ (Santa Monica, California)
(Names of ALL Applicants)

for USE OF CYTOKINES, CELLS, AND MITOGENS TO INHIBIT GRAFT VERSUS HOST DISEASE
(Title of Invention)

This __ continuation __ divisional X continuation-in-part

claims priority to applications Serial No. 60/151,987, filed on September 1, 1999 (pending); Serial No. 09/261,890 filed on March 3, 1999 (pending); and Serial No. 60/076,677 filed on March 3, 1998.

1. (a) __ Enclosed is a new application.
   (b) X Enclosed is a continuation-in-part application.
   (c) __ Enclosed is a copy of the prior application.

2. (a) __ Enclosed is a new Declaration.
   (b) __ Enclosed is a copy of the prior Declaration as originally filed.

3. (a) __ Enclosed is a Small Entity Affidavit.
   (b) __ A Small Entity Affidavit is of record in the prior application.

4. X The filing fee is calculated below:

Claims as filed in the prior application, less any claims canceled by amendment below:

	(Col. 1) NO. FILED	(Col. 2) NO. EXTRA	SMALL ENTITY RATE	SMALL ENTITY FEE	OTHER THAN SMALL ENTITY RATE	OTHER THAN SMALL ENTITY FEE
BASIC FEE				**$345**		**$690**
TOTAL CLAIMS	45 - 20 =	25	× 9 =	$225	× 18 =	$
INDEP CLAIMS	5 - 3 =	2	× 39 =	$78	× 78 =	$
MULTIPLE DEPENDENT CLAIM PRESENTED	X yes __ no		+130 =	$130	+260 =	$
If the difference in Col 1 is less than zero, enter "0" in Col. 2			TOTAL	**$778**	TOTAL	**$**

6. X The filing fee is ***NOT*** being submitted with this transmittal letter.

7. __ Cancel in this application original claims _____ of the prior application before calculating the filing fee. (At least one independent claim must be retained for filing purposes.)

8. __ Amend the specification by inserting before the first line the sentence:

--This is a __ continuation __ divisional __ continuation-in-part

of application Serial No. _____ filed _____.--

9. (a) X Informal drawings are enclosed (17 sheets).
(b) __ Formal drawings are enclosed.

10. (a) __ Priority of applications Serial No. 60/151,987, filed on September 1, 1999 (pending) in the United States of America; Serial No. 09/261,890 filed on March 3, 1999 (pending) in the United States of America; and Serial No. 60/076,677 filed on March 3, 1998 in the United States of America is claimed under 35 U.S.C. 119/120.
(b) __ The certified copy has been filed in prior application Serial No. _____ filed on _____.

11. __ The prior application is assigned of record to _____

12. __ The power of attorney in the prior application is to:

Name: _____
Address _____
_____
_____

(a) __ The power appears in the original papers in the prior application.
(b) __ Since the power does not appear in the original papers, a copy of the power in the prior application is enclosed.
(c) __ A new power has been executed and is enclosed.
(d) X Address all future communications to:

ROBIN M. SILVA

FLEHR HOHBACH TEST ALBRITTON & HERBERT LLP
Four Embarcadero Center - Suite 3400
San Francisco, California 94111-4187
Tel.: (415) 781-1989
Fax: (415) 398-3249

13. __ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

14. __ I hereby verify that the attached papers are a true duplicate of prior application Serial No. _____ as originally filed on _____.

Date: 9/1/00 Signature: Robin M. Silva
ROBIN M. SILVA, Reg. No. 38,304

Address of Signer:

FLEHR HOHBACH TEST
ALBRITTON & HERBERT LLP
4 Embarcadero Center - Suite 3400
San Francisco, California 94111-4187
Tel.: (415) 781-1989
Fax: (415) 398-3249

__ Attorney or agent of record

X Filed under Section 1.34(a)

1028341

USE OF CYTOKINES, CELLS, AND MITOGENS TO INHIBIT GRAFT VERSUS HOST DISEASE

This application is a continuing application of U.S.S.N.s 60/151,987, filed September 1, 1999, 09/261,890, filed March 3, 1999 and 60/076,677, filed March 3, 1998.

FIELD OF THE INVENTION

The field of the invention is generally related to pharmaceutical agents useful in treating graft-versus-host disease (GVHD) in patients that have received allogenic bone marrow transplants.

BACKGROUND OF THE INVENTION

Organ transplantation is now used with great success to improve the quality of human life. Substantial progress has been made in using kidneys, hearts, and livers from unrelated individuals. However, transplantation of hematopoietic stem cells from an unrelated (or allogeneic) donor is a more complicated endeavor. Here multipotent stem cells which have the capacity to regenerate all the blood-forming elements and the immune system are harvested from bone marrow or peripheral blood from one individual are transferred to another. However, histocompatibility differences between donor and recipient results in a higher incidence of transplant-related complications, and has limited the use of this procedure (Forman et al., *Blackwell Scientific Publications*, 1994).

It is unfortunate that only a few individuals are candidates for allogeneic hematopoietic stem cell transplantation at the present time because the spectrum of diseases treatable by this procedure has steadily increased. These diseases now include hematologic malignancies such as the acute or chronic leukemias, multiple myeloma, myelodysplastic syndromes; lymphomas; and the severe anemias such as aplastic anemia or thalassemia.

Allogeneic stem cell transplantation begins with treatment of the recipient with a highly immunosuppressive conditioning regimen. This is most commonly accomplished with high doses of

chemotherapy and radiation which effectively kill all the blood forming elements of the bone marrow. Besides preparing the recipient bone marrow for donor stem cell transplantation, the conditioning regimen serves to kill much of the malignancy that remains in the body. The period of time between the completion of the conditioning regimen, and engraftment of the donor stem cells is the most dangerous for the recipient. It is during this time that the patient is completely immunocompromised and susceptible to a host of life-threatening infections. This vulnerability persists until the grafted donor stem cells proliferate and differentiate into the needed white blood cells and immune cells needed to combat infections.

Moreover, donor stem cell preparations generally contain immune cells called T lymphocytes. Unless the donor stem cells originate from an identical twin the transferred T cells turn against the recipient's tissues and trigger a deadly ailment called graft versus host disease (or GVHD). This is because the donor T lymphocytes recognize histocompatibility antigens of the recipient as foreign and respond by causing multi-organ dysfunction and destruction.

Current techniques of immunosuppression have made allogeneic stem cell transplantation from a related, histocompatible (HLA-matched) donor much safer than it once was. Allogeneic stem cell transplantation from an unrelated, HLA-matched donor is commonly complicated by serious, often fatal GVHD. The threat of GVHD is even higher when the stem cell donor is HLA mismatched.

Since only 30% of patients in need of allogeneic stem cells will have a sibling with identical histocompatibility antigens (Dupont, B., *Immunol Reviews 157*:12, 1997), there is a great need to make HLA-matched unrelated, and HLA-mismatched transplantation a safer procedure. There have been two principal approaches to resolving this problem. The first has been to deplete the graft of contaminating T lymphocytes and the second has been to inactivate the T cells so they cannot attack the recipient.

In the 1970's it became evident that ex-vivo removal of mature T lymphocytes from a bone marrow graft prior to transplantation dramatically decreased or prevented GVHD in animals receiving marrow grafts across major histocompatibility barriers (Rodt, H. *J. Immunol 4*:25-29, 1974; and 4 Vallera et al., *Transplantation 31*:218-222, 1981). However, with T cell depletion the incidence of graft failure, graft rejection, relapse of leukemia, and viral-induced lympho-proliferative disease markedly increased (Martin et al. *Blood 66*:664-672, 1985; 6 Patterson et al. *Br J Hematol 63*:221-230, 1986; Goldman et al. *Ann Intern Med 108*:806-814, 1988; and Lucas et al. *Blood 87*:2594-2603, 1996). Thus, the transplantation of donor T cells on the stem cells has beneficial as well as deleterious effects. One needs the facilitating effect of the T cells on the engraftement of stem cells and the now well recognized graft-versus-tumor effects, but not graft-versus host disease.

Several approaches have been used to decrease T cell activation. These include: 1) *in vivo* immunosuppressive effects of drugs such as FK506 and rapamycin (Blazar et al. *J. Immunol 153*:1836-1846, 1994; Dupont et al. *J. Immunol 144*:251-258, 1990; Morris, *Ann NY Acad Sci 685*:68-72. 1993; and Blazar et al. *J Immunol 151*:5726-5741, 1993); 2) the *in vivo* targeting of GVHD-reactive T cells using intact and F(ab')2 fragments of monoclonal antibodies(mAb)reactive against T cell determinants or mAb linked to toxins (Gratama et al. *Am j Kidney Dis 11*:149-152, 1984; Hiruma et al. *Blood 79*:3050-3058, 1992; Anasetti et al. *Transplantation 54*:844-851, 1992; Martin et al. *Bone Marrow Transplant 3*:437-444, 1989); 3) inhibition of T cell signaling via either IL-2/cytokine receptor interactions (Herve et al. *Blood 76*:2639-2640, 1990) or the inhibition of T cell activation through blockade of co-stimulatory or adhesogenic signals (Boussiotis et al. *J Exp Med 178*:1753-1763, 1993; Gribben et al. *Blood 97*:4887-4893, 1996; and Blazar et al. *Immunol Rev 157*:79-90, 1997); 4) the shifting of the balance between acute GVHD-inducing T helper-type 1 T cells to anti-inflammatory T helper-type 2 T cells via the cytokine milieu in which these cells are generated (Krenger et al. *Transplantation 58*:1251-1257, 1994; Blazar et al. *Blood 88*:247, 1996, abstract; Krenger et al. *J Immunol 153*:585-593. 1995; Fowler et al. *Blood 84*:3540-3549, 1994); 5) the regulation of alloreactive T cell activation by treatment with peptide analogs which affect either T cell receptor/major histocompatibility complex (MHC) interactions, class II MHC/CD4 interactions, or class I MHC/CD8 interactions (Townsend and Korngold (unpublished data)); and 6) the use of gene therapy to halt the attack of donated cells on the recipient's tissues (Bonini et al. *Science 276*:1719-24, 1997).

There is suggestive evidence that the T lymphocytes from non-identical donors can become tolerant to the recipient's tissues. Unlike patients who receive solid organ allografts for whom life-long immunosuppressive therapy is needed to control chronic rejection, there is evidence of immunologic tolerance with stem cell allografts. The majority of these patients can be withdrawn from immune suppression without further evidence of GVHD (Storb et al. *Blood 80*:560-561, 1992; and Sullivan et al. *Semin Hematol 28*:250-259, 1992).

Immunologic tolerance is a specific state of non-responsiveness to an antigen. Immunologic tolerance generally involves more than the absence of an immune response; this state is an adaptive response of the immune system, one meeting the criteria of antigen specificity and memory that are the hallmarks of any immune response. Tolerance develops more easily in fetal and neonatal animals than in adults, suggesting that immature T and B cells are more susceptible to the induction of tolerance. Moreover, studies have suggested that T cells and B cells differ in their susceptibility to tolerance induction. Induction of tolerance, generally, can be by clonal deletion or clonal anergy. In clonal deletion, immature lymphocytes are eliminated during maturation. In clonal anergy, mature lymphocytes present in the peripheral lymphoid organs become functionally inactivated.

Following antigenic challenge stimulation, T cells generally are stimulated to either promote antibody

production or cell-mediated immunity. However, they can also be stimulated to inhibit these immune responses instead. T cells with these down-regulatory properties are called "suppressor cells".

Although it is known that T suppressor cells produce cytokines such as transforming growth factor beta (TGF-beta), interleukin 4 (IL-4) or interleukin (IL-10) with immunosuppressive effects, until recently the mechanisms responsible for the generation of these regulatory cells have been poorly understood. It was generally believed that CD4+ T cells induce CD8- T cells to develop down-regulatory activity and that interleukin 2 (IL-2) produced by CD4+ cells mediates this effect. Although most immunologists agree that IL-2 has an important role in the development of T suppressor cells, whether this cytokine works directly or indirectly is controversial (Via et al. *International Immunol 5*:565-572, 1993; Fast, *J Immunol 149*:1510-1515, 1992; Hirohata et al. *J Immunol 142*:3104-3112, 1989; Taylor, *Advances Exp Med Biol 319*:125-135, 1992; and Kinter et al., *Proc. Natl. Acad. Sci. USA 92*:10985-10989, 1995). Recently, IL-2 has been shown to induce CD8+ cells to suppress HIV replication in CD4-T cells by a non-lytic mechanism. This effect is cytokine mediated, but the specific cytokine with this effect has not been identified (Barker et al. *J Immunol 156*:4476-83, 1996; and Kinter et al. *Proc Natl Acad Sci USA 99*:10985-9 1995).

A model using human peripheral blood lymphocytes to study T cell/B cell interactions in the absence of other accessory cells has been developed (Hirokawa et al. *J. Immunol. 149*:1859-1866, 1992). With this model it was found that CD4+ T cells by themselves generally lacked the capacity to induce CD8+ T cells to become potent suppressor cells. The combination of CD8+ T cells and NK cells, however, induced strong suppressive activity (Gray et al. *J Exp Med 180*:1937-1942, 1994). It was then demonstrated that the contribution of NK cells was to produce TGF-beta in its active form. It was then reported that a small non-immunosuppressive concentration (10-100 pg/ml) of this cytokine served as a co-factor for the generation of strong suppressive effects on IgG and IgM production (Gray et al. *J Exp Med 180*:1937-1942, 1994). Further, it was demonstrated that NK cells are the principal lymphocyte source of TGF-beta (Gray et al. *J Immunol*, 160:2248-2254,1998).

TGF-beta is a multifunctional family of cytokines important in tissue repair, inflammation and immunoregulation (Border et al. *J Clin Invest 90*:1-7, 1992; and Sporn et al. *J Cell Biol 105*:1039-1045, 1987). TGF-beta is unlike most other cytokines in that the protein released is biologically inactive and unable to bind to specific receptors (Massague, *Cell 69*:1067-1070, 1992). The conversion of latent to active TGF-beta is the critical step which determines the biological effects of this cytokine.

There is some evidence that NK cell-derived TGF-beta has a role in the prevention of GVHD. Whereas the transfer of stem cells from one strain of mice to another histocompatibility mismatched strain resulted in death of all recipients from GVHD within 19 days, the simultaneous transfer of NK cells from the donor animals completely prevented this consequence. All the recipient mice survived

indefinitely. This therapeutic effect, however, was completely blocked by antagonizing the effects of TGF-beta by the administration of a neutralizing antibody (Murphy et al. *Immunol Rev 157*:167-176, 1997).

It is very likely, therefore, that the mechanism whereby NK cell-derived TGF-beta prevented GVHD is similar to that described by Horwitz et al., in the down-regulation of antibody production (Horwitz et al., (1998) Arthritis. Rheum., 41:838-844). In each case NK cell-derived TGF-beta was responsible for the generation of suppressor lymphocytes that blocked these respective immune responses. The mouse study is of particular interest since the histocompatibility differences between genetically disparate inbred mice strains would mirror that of unrelated human donors. A modification of this strategy, therefore might overcome GVHD in mismatched humans.

Anti-CD2 monoclonal antibodies and other constructs that bind to the CD2 co-receptor have been shown to be immunosupressive. It has now been demonstrated that at least one mechanism to explain this immunosuppressive effect is by inducing the production of TGF-beta (Gray et al. *J Immunol*, 160:2248-2254, 1998).

One strategy to prevent GVHD would be to isolate and transfer NK cells along with the stem cells. Another would be to treat the immunocompromised recipient who has received allogeneic stem cells with TGF-beta, anti-CD2 monoclonal antibodies, IL-2 or a combination of these cytokines. The first strategy would be difficult because NK cells comprise only 10 to 20% of total lymphocytes so that it would be difficult to harvest a sufficient number of cells for transfer. The second strategy is limited by the systemic toxic side effects of these monoclonal antibodies and cytokines. IL-2 and TGF-beta have numerous effects on different body tissues and are not very safe to deliver to a patient systemically. What is needed, therefore, is a way to induce mammalian cells to suppress the development of GVHD *ex vivo*.

An approach which generates regulatory T cells ex vivo would provide an effective means of suppressing the development of GVHD. A first step towards achieving the goal of generating regulatory T cells ex vivo is to identify T cells which have potent suppressive effects. There is compelling evidence that in addition to CD8+ cells, certain CD4+ cells have potent suppressive effects. This evidence is based upon the following observations. The first is that injection of peripheral T cells from normal mice depleted of $CD45RB^{hi}$ CD4+ CD25+ T cells into athymic mice results in a high incidence of organ-specific autoimmune disease (Powrie F, et al., *J Exp Med* 183:2669-74, 1996). The second is that certain strains of neonatally thymectomized mice develop multi-organ specific autoimmunity (Powrie F, et al., *J Exp Med* 183:2669-74, 1996; Sakaguchi, S., et al., *J Exp Med* 161(1):72-87, 1985). Taken together, these observations suggest that the normal immune system contains autoreactive T cells capable of inducing severe autoimmune disease, but

that this result can be prevented by regulatory T cells. This suggestion has been confirmed by the observation that the autoimmune syndromes described above were prevented by the transfer of CD4+ CD25+ T cells (Asano, M., et al., *J Exp Med* 184:387-396, 1996; Sakaguchi, S, et al., *J Immunol* 155:1151-1164, 1995; Read, et al., J. Exp. Med., 192(2):295-302, 2000). These CD4+ CD25+ T cells differentiate in the thymus and are exported to the periphery where they suppress the activation of potentially self-reactive cells (Asano, M., et al., *J Exp Med* 184:387-396, 1996; Sakaguchi, S, et al., *J Immunol* 155:1151-1164, 1995; Papiernik, M., et al., *J. Immunol.* 158:4642-4653, 1997; Suri-Payer, E., et al., *J. Immunol.* 160:1212- 1218, 1998; Jackson, A. L., et al., *Clin. Immunol. Immunopathol.* 54(1):126-133, 1990; Kanegane, H., et al., *Int. Immunnol.* **3**(12):1349-1356, 1991). Neonatal mice lack CD4+ CD25+ cells because they are not produced until 1 week after birth (Asano, M., et al., *J Exp Med* 184:387-396, 1996; Suri-Payer E, et al., Eur. J. Immunol 29:669-677, 1999).

Thus, CD4+ CD25+ T cells are potent inhibitors of polyclonal T cell activation (Thornton, A.M., and Shevach, E.M., *J. Exp. Med.* 188:287-296, 1998). After activation via the T cell receptor (TCR), they inhibit IL-2 production by the responding T cells (Takahashi, T., et al., *Int. Immunol.* 10:1969-80, 1998; Thornton, A.M., and Shevach, E.M., *J. Immunol.* 164(1):183-190, 1999). Unlike other regulatory T cells, which produce inhibitory cytokines (Powrie F, et al., *J Exp Med* 183:2669-74, 1996; Weiner, H.L., et al., *Annu. Rev. Immunol.* 12:809-837, 1994), these cells suppress immune responses by a contact-dependent mechanism, at least *in vitro* (Thornton, A.M., and Shevach, E.M., *J. Immunol.* 164(1):183-190, 1999). Others have described CD4+ CD25+ cells that regulate anergy in neonatally tolerized mice (Gao, Q., et al., *Transplantation.* 68:1891-1897, 1999).

We have found that regulatory CD4+ CD25+ T cells can be generated in the periphery, as well as the thymus, and that TGF-β directs naive CD4+ cells to develop this property. Therefore, in addition to its well described immunosuppressive effects, TGF-β has positive effects on the growth and development of T cells. TGF-β plays a critical role in the differentiation of both CD8+ T cells and CD4+ T cells to become suppressor cells.

Accordingly, a strategy to generate suppressor T cells ex vivo which are hardy and able to proliferate upon introduction into a recipient is desirable. Such a strategy would involve immunizing CD4+ cells against the white blood cells of an unrelated individual in the presence of TGF-β. CD4+ cells immunized in this manner are activated to become CD25+ and develop suppressive properties similar, if not identical, with the naturally occurring thymus-derived CD4+ CD25+ cells. Following stem cell transplantation, these CD4+ CD25+ suppressor cells would be repeatedly re-stimulated by the donor hematopoietic cells and, thus, have long term inhibitory effects.

SUMMARY OF THE INVENTION

In accordance with the objects outlined above, the present invention provides methods for inducing T cell tolerance in a sample of *ex vivo* peripheral blood mononuclear cells (PBMCs) comprising adding a suppressive-inducing composition to the cells. The suppressive-inducing composition can be IL-2, IL-10, TGF-β, or a mixture.

In an additional aspect, the present invention provides methods for treating donor cells to ameliorate graft versus host disease in a recipient patient. The methods comprise removing peripheral blood mononuclear cells (PBMC) from a donor, and treating the cells with a suppressive-inducing composition for a time sufficient to induce T cell tolerance. The cells are then introduced into a recipient patient. The PBMCs can be enriched for CD8+ cells or CD4+, if desired. The methods may additionally comprising adding the treated cells to donor stem cells prior to introduction into the patient.

In an additional aspect, the present invention provides methods for activating T cells to become suppressor cells. The methods comprise treating T cells with TGF-β. The methods may additionally comprise treating T cells with TFG-β in combination with an activating agent.

In an additional aspect, the invention provides kits for the treatment of donor cells comprising a cell treatment container adapted to receive cells from a donor and at least one dose of a suppressive-inducing composition. The kits may additionally comprise written instructions and reagents. The cell treatment container may comprise a sampling port to enable the removal of a fraction of the cells for analysis, and an exit port adapted to enable at least a portion of the cells to be transported to a recipient patient.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B depict that TGF-β can upregulate expression of CD40 Ligand (CD40L) on T cells. Purified T cells were stimulated with PMA (20 ng/ml) and ionomycin (5 μM) in the presence or absence of TGF-β. After 6 hours the cells were stained with anti-CD40L antibodies. In the absence of TGF-β, there were 30% positive cells (solid line, panel A). With 100 pg/ml of TGF-β, 66% of the cells were positive (solid line, panel B). The dotted line in both panels is the reactivity of a control antibody.

Figures 2A, 2B, 2C and 2D depict that TGF-β increases TNF-α expression by CD8+ cells. Purified CD8+ cells were stimulated for 24 hours with Con A (5 μg/ml) ± TGF-β (10 pg/ml) ± IL-2 (10 U). During the last 6 hours, monensin (2 μM) was also present to prevent cytokine release. The cells were first stained with anti-CD69 to distinguish the activated cells. Then the cells were fixed (4% paraformaldhyde), permeabilized (0.1% saponin) and stained with anti-TNF-α antibodies.

Figures 3A and 3B depict TGF-β enhances IL-2 expression by T cells. Purified T cells were stimulated in the presence or absence of TGF-β (1 ng/ml). In the absence of TGF-β, 36% of the cells were positive (panel A, solid line) whereas with TGF-β, 53% were positive (panel B, solid line).

Figures 4A, 4B, and 4C depict that TGF-β can enhance or inhibit cytotoxic activity. Panel A shows the ability of donor T cells to recognize and kill recipient blood cells. In panel B, purified T cells were cultured with irradiated allogenic stimulator cells in the presence or absence of the indicated cytokines. After 48 hours, the cells were washed and after a further 3 days, assayed for cytotoxic activity against ^{31}Cr-labelled sitmulator ConA blasts. In panel C, purified CD8+ cells were conditioned with the indicated amounts of TGF-β (10 pg/ml). After 48 hours, the cells were washed. After 5 days of culture, cytotoxic activity was determined.

Figure 5 depicts the suppression of cytotoxic T cell activity against mismatched human cells by CD8+ T cells conditioned by TGF-β. Purified CD8+ cells from donor A were cultured with irradiated T cell-depleted mononuclear cells from unrelated donor B for 48 hours with our without TGF-β at 10 pg/ml. The cells were then washed and added to T cells from donor A and irradiated non-T cells from donor B and cultured for an additional 5 days. Cytotoxicity by donor A T cells against chromium labeled ConA blasts of donor B was measured by a conventional 4 hour chromium release assay. Various effector to target ratios are shown. Open circles: T cells from donor A and non-Te cells from donor B without added CD8+ cells. Closed squares: control CD8+ T cells added. Closed circles: TGF-β treated CD8+ cells added.

Figure 6 illustrates that naive CD4+ T cells develop potent suppressive activity when activated in the presence of TGF-β. In a two step co-culture procedure, purified T cell subsets from donor A were stimulated for 5 days with irradiated (3000 cGy) allogeneic stimulator cells from donor B ± TGF-β (1 ng/ml). The secondary cultures consisted of mixing the primed T cell subsets with fresh syngeneic T cells in a 1:4 ratio and culturing these cells with irradiated stimulator cells from donor B for 5 days. CTL activity against Con A blasts from donor B was then determined in a standard four hour chromium release assay at the effector to target cell ratios indicated. Figure 6A depicts alloactivated CD4+ CD45RA+ cells primed with TGF-β markedly suppressed the generation of CTL activity in comparison with control CD4+ cells. Additional controls not shown were CD4+ cells cultured for 5 days with TGF-β, but without allogeneic MHC stimulation. These cells had no suppressive effects. In Figure 6B, the experimental protocol was similar except that CD8+ CD45RA+ cells were substituted for CD4+ CD45RA+ cells. Under these conditions, priming CD8+ CD45RA+ cells with alloantigens in the presence of TGF-β did not enhance the development of suppressive activity.

Figure 7 depicts the effect of CD4 cells primed with TGF-beta on allo-cytotoxic T lymphocyte (CTL) activity. The addition of CD4 CD45RA cells that had been cultured for 5 days without stimulators had

no effect on CTL activity (result not shown). Culturing these T cells with stimulator cells resulted in modest to moderate suppressive activity. In all experiments, culture of these T cells with TGF-beta 1 ng/ml markedly suppressed, or abolished allo-CTL activity.

Figure 8 demonstrates that regulatory T cells require cell contact to inhibit CTL activity. CD4reg and control CD4+ cells were generated by activating naive CD4+ cells with allogeneic stimulator cells ± TGF-β as described above. In secondary cultures, CD4reg or control CD4+ cells were either directly mixed with the responder cells or separated from them by a semipermeable membrane using Transwell™ chambers. The Transwells contained CD4+ cells and stimulator cells. The results shown are one of 3 independent experiments.

Figure 9 depicts that activation of naive CD4+ T cells in the presence of TGF-β enables them to respond more vigorously to alloantigens, accelerates their conversion to the activated phenotype, and increases their viability. Purified naive CD4+ T cells were cultured for 5 days without stimulator cells [gray shaded area], with irradiated allogeneic stimulator cells [thin dotted line], or with stimulatory cells and TGF-β (1 ng/ml) [thick line], and expression of cell surface or intracellular antigens were determined by flow cytometry. Cells (10^5) were labeled with FITC-conjugated (anti-CD4, anti-CD25), phycoerythrin-conjugated (anti-CD45RA, anti-CTLA-4) or CyChrome-conjugated (anti-CD4) mAbs. CTLA-4 expression was analyzed by intracellular staining. To detect apoptotic cells, annexin-V staining was performed according to the manufacturer's instructions. This experiment is representative of at least 5 studies with each marker.

Figure 10 shows that CD4reg display CD25 and require remarkably small numbers for potent suppressive activity. Naive CD4+ T cells primed with irradiated allogeneic stimulator cells ± TGF-β (1 ng/ml) and CD4reg were separated into CD25+ and CD25- fractions by cell sorting. Figure 10A depicts the effect of primed CD4+ cells mixed with fresh T cells at a 1:4 ratio. Figure 10B depicts the effect of various dilutions of these primed CD4+ T cells added to fresh responder cells on the generation of CTL activity. Results shown were performed at an effector to target cell ratio of 100:1. The results shown are representative of 3 similar experiments.

Figure 11 shows that CD4+ CD25+ T cells can be expanded and retain their potent suppressive effects. The CD4+ CD25+ fraction of naive CD4+ cells primed in the presence of TGF-β were obtained by cell sorting and cultured in the presence of IL-2 (10 U/ml) for 5 days. The percentages indicated were added to fresh T cells and examined for inhibition of the generation of CTL activity. Irradiated CD4+ CD25+ cells served as controls.

Figure 12 depicts suppression of lymphocyte proliferation by regulatory CD4+ T cells induced with TGF-β. Naïve CD4+ T cells from donor A were mixed with stimulator cells as described above and

added to fresh responder and stimulator cells at the indicated ratios. The bars show the uptake of tritiated thymidine ± SEM after 7 days of culture. The lightly shaded bar (Nil) indicates the proliferative response of the responder T cells without added CD4+ cells. The darkly shaded bar indicates the effect of control CD4+ cells which had been cultured with stimulator cells without TGF-β. The black bar indicates the effect of CD4+ cells that had been mixed with stimulator cells in the presence of TGF-β (1 ng/ml). The effect of these CD4+ cells on the proliferative response of fresh responder cells added to irradiated stimulator cells after 7 days of culture is shown. The bars indicate the mean uptake of tritiated thymidine.

Figure 13 depicts the regulatory activity of CD25+ CD4 T cells. CD4+ cells were stimulated with irradiated allogeneic non-T cells ± TGF-β (1 ng/ml) for 5 days. After washing, the CD4+ cells were stained with DII and fresh responder T cells were stained with carboxyfluorescein (CFSE). control or TGF-β primed CD4+ cells were added to the responder T cells and allo-stimulator cells in a 1:4 ratio. After 5 days, the cells were harvested and analyzed by flow cytometry. The intensity of CFSE in CD8+ cells was determined by gating on DII negative cells. Note that the addition of TGF-β primed CD4+ cells to responder T cells markedly decreased cell division by CD8+ cells.

Figure 14 cytotoxic T cell activity depicts that CD4reg block the proliferative response of responder T cells to alloantigens and CD8+ T cells are their principal target. In Figure 14A, CD4reg or primed control CD4+ cells were mixed with fresh T cells in a 1:4 ratio (10^5/well) and cultured in triplicate with irradiated stimulator cells (10^5/well) for 5 days. The cultures were pulsed with [^{3}H]TdR for the last 18 hours. In Figure 14B, the phenotype of the cultured CD8+ cells was determined by flow cytometry gating on CD8+ cells. The gray shaded area indicates cells cultured for 5 days without stimulator cells. The thick line indicates CD8 cells cultured with stimulator cells and CD4reg. The thin dotted line indicates alloactivated CD8 cells cultured with control CD4 cells. The lymphocytes (10^5) were labeled with FITC-conjugated (anti-CD69), phycoerythrin-conjugated (anti-CD25, anti-CD95) or CyChrome-conjugated (anti-CD8) mAbs. Annexin-V straining, CFSE levels and DNA content by propidium iodide staining are also shown.

Figure 15 depicts that repeated stimulation of T cells with a low dose of staphylococcal enterotoxin B (SEB) induces T cells to produce immunosuppressive levels of TGF-β. CD4+ T cells were stimulated with SEB (0.01ng/ml) and irradiated B cells as superantigen presenting cells with our without TGF-β at the times indicated by the arrows. Active TGF-β was measured 2 or 5 days later.

Figure 16 depicts that repeated stimulation of CD4+ T cells with a low dose of SEB enables these cellls to produce immunosuppressive levels of TGF-β. CD4+ T cells were stimulated with SEB (0.01 ng/ml) and irradiated B cells as superantigen presenting cells with or without TGF-β at the times indicated by the arrows. Active TGF-β was measured 2 or 5 days later.

Figure 17 shows the effects of SEB on naive (CD45RA+ CD45RO-) CD4+ and CD8+ T cells. The cells were stimulated with SEB every 5th day for a total of three stimulations. The percentages of each T cell subset and the cells expressing the CD25 IL-2 receptor activation marker were determined after each stimulation. Panels A and C show that if TGF-β 1ng/ml was included in the initial stimulation, CD4+ T cells became the predominant subset in the cultures after repeated stimulation. Panels B and D show that CD25 expression by SEB stimulated cells decreases by the third stimulation in control cultures. However, CD25 expression remains high if the T cells have been primed with TGF-β.

DETAILED DESCRIPTION OF THE INVENTION

The present invention allows for the transfer of histoincompatible stem cells to humans with a variety of malignant or hereditary diseases using a method to prevent life-threatening graft-versus-host disease. This is accomplished by treatment of donor cells with a combination of alloantigens and/or cytokines ex vivo. The particular advantage of this procedure is that it avoids the removal of donor T cells which facilitate stem cell engraftment and that have the potential to attack any remaining malignant cells. Once a state of tolerance between donor and host has been achieved, non-conditioned donor T cells can be transferred to maximize the beneficial graft-versus-tumor immune response.

This strategy is unlike almost all other treatment modalities currently in use. These cytokines and mitogens described would have severe toxic side effects if administered *in vivo*. The ex-vivo protocol described avoids these side effects. The ability to successfully engraft histoincompatible stem cells for treatment of life-threatening diseases would be a milestone in medicine.

In addition, a further advantage of the present invention is that it may avoid or minimize the very toxic immunosuppressive medicines that must be given to the recipient to prevent GVHD. These medicines also block the ability of the donor-derived lymphocytes which repopulate the immune system of the recipient from becoming "educated" to their new host. Therefore, it is difficult to stop the immunosuppressive drugs, unless an alternative treatment such as the present invention is used.

The strategy of the present invention is to suppress GVHD by inhibiting the T cell response of donor T cells to the recipient's transplantation antigens, and inducing a tolerant state in the donor cells. This result prevents the donor cells from attacking recipient cells. Surprisingly, the methods outlined herein result in not only the suppression of the treated cells, but additionally induces some CD8+ and CD4+ T cells to prevent other donor T cells from killing recipient cells as well, i.e. they become tolerant. That is, the methods outlined herein not only decrease the capacity of the donor's cells to attack the recipient's cells, but induces some of the donor's cells to assume a surveillance role and prevent other

donor cells from mounting an immune attack against the recipient host. The net result is for the donor lymphocytes to be tolerant to the histocompatibility antigens of the recipient, but does not impair the ability of the new lymphocytes to attack tumor cells.

Another significant potential advantage of this strategy is a low probability of serious adverse side effects. Since only trace amounts of suppressive-inducing compositions such as cytokines will be returned to the patient, there should be minimal toxicity.

Accordingly, the present invention is drawn to methods of treating donor cells for transplantation into a recipient that comprise removing peripheral blood mononuclear cells (PBMCs) from the donor and treating the cells with a composition that is on one hand suppressive, but on the other hand generates surveillance cells to prevent an immune attack.

The present invention shows that the treatment of the donor cells with a suppressive-inducing composition blocks an immune attack against the recipient's cells. Without being bound by theory, it appears that there are several ways the methods of the invention may work. First of all, the donor cells are activated to become tolerant to the recipient's cells. Secondly, the donor cells are activated to become regulatory cells, that is, as regulatory cells they act to prevent other donor cells from proliferating in response to the recipient's cells and killing them. These results lead to amelioration of a GVHD response. Without being bound by theory, it appears that the inhibition of cytotoxic activity may occur as a result of inhibitory cytokines that are produced by the regulatory T cells such as TGF-β on the cells or a contact-dependent mechanism as depicted in the Figures.

Thus, in a preferred embodiment, the present invention induces tolerance in the donor cells to recipient tissue, thus avoiding GVHD, by treating them with a suppressive-inducing composition *ex vivo*.

Accordingly, the present invention provides methods of treating donor cells to induce or establish tolerance to recipient cells prior to transplantation into a recipient patient to decrease or eliminate a graft-versus-host response. By "T cell tolerance" herein is meant the failure of T cells to mount a harmful immune response against other cells. This can be due to the failure of T cells to proliferate in response to antigens, or failure to become cytotoxic T cells. Without being bound by theory, this may be due to anergy or death of the T cells. Preferably, the T cells retain the ability to recognize other antigens as foreign, to facilitate tumor killing and general immunological responses to foreign antigens.

Using the methods of the present invention, tolerance is established by treating a population of T cells *ex vivo* with a suppressive-inducing composition. Regulatory cells are generated by treating a population of T cells *ex vivo* with an activating agent and a suppressive-inducing composition.

Using the methods outlined herein, a GVHD response is suppressed or treated. By "treating" GVHD herein is meant that at least one symptom of the GVHD is ameliorated by the methods outlined herein. This may be evaluated in a number of ways, including both objective and subjective factors on the part of the patient as is known in the art. For example, GVHD generally exhibits a skin rash, an abnormality in liver function studies, fever, general symptoms including fatigue, anemia, etc.

By "patient" herein is meant a mammalian subject to be treated, with human patients being preferred. In some cases, the methods of the invention find use in experimental animals and in the development of animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters; and primates.

The methods provide for the removal of blood cells from a patient. In general, peripheral blood mononuclear cells (PBMCs) are taken from a patient using standard techniques. By "peripheral blood mononuclear cells" or "PBMCs" herein is meant lymphocytes (including T-cells, B-cells, NK cells, etc.) and monocytes. As outlined more fully below, it appears that the main effect of the suppressive-inducing composition is to enable CD8+ T or CD4+ T cells to become tolerant and to develop the capacity to render other T cells tolerant. Accordingly, the PBMC population should at least comprise CD8+ or CD4+ T cells.

Preferably, only PBMCs are taken, either leaving or returning red blood cells and polymorphonuclear leucocytes to the patient. This is done as is known in the art, for example using leukophoresis techniques. In general, a 5 to 7 liter leukophoresis step is done, which essentially removes PBMCs from a patient, returning the remaining blood components. Collection of the cell sample is preferably done in the presence of an anticoagulant such as heparin, as is known in the art.

In some embodiments, as is outlined in Example 7, no leukophoresis step is required. For example, a population of naive CD4+ T cells, when stimulated in the presence of TGF-β, can expand, as shown in Figure 11.

In general, the sample comprising the PBMCs can be pretreated in a wide variety of ways. Generally, once collected, the cells can be additionally concentrated, if this was not done simultaneously with collection or to further purify and/or concentrate the cells. The cells may be washed, counted, and resuspended in buffer transferred to a sterile, closed system for further purification and activation.

The PBMCs are generally concentrated for treatment, using standard techniques in the art. In a preferred embodiment, the leukophoresis collection step results in a concentrated sample of PBMCs, in a sterile leukopak, that may contain reagents or doses of the suppressive composition, as is more fully outlined below. Generally, an additional concentration/purification step is done, such as Ficoll-

Hypaque density gradient centrifugation as is known in the art. Separation or concentration procedures include but are not limited to magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents, either joined to a monoclonal antibody or used with complement, "panning", which uses a monoclonal antibody attached to a solid matrix. Antibodies attached to solid matrices, such as magnetic beads, agarose beads, polystyrene beads, follow fiber membranes and plastic surfaces, allow for direct separation. Cells bound by antibody can be removed or concentration by physically separating the solid support from the cell suspension. The exact conditions and procedure depend on factors specific to the system employed. The selection of appropriate conditions is well within the skill in the art.

Antibodies may be conjugated to biotin, which then can be removed with avidin or streptavidin bound to a support, or fluorochromes, which can be used with a fluorescence activated cell sorter (FACS), to enable cell separation. Any technique may be employed as long as it is not detrimental to the viability of the desired cells.

In a preferred embodiment, the PBMCs are separated in a automated, closed system such as the Nexell Isolex 300i Magnetic Cell Selection System. Generally, this is done to maintain sterility and to insure standardization of the methodology used for cell separation, activation and development of suppressor cell function.

Once purified or concentrated the cells may be aliquoted and frozen, preferably, in liquid nitrogen or used immediately as described below. Frozen cells may be thawed and used as needed.

Cryoprotective agents, which can be used, include but are not limited to dimethyl sulfoxide (DMSO) (Lovelock, J. E. and Bishop, M. W. H., 1959, Nature 183:1394-1395; Ashwood-Smith, M. J., 1961, Nature 190:1204-1205), hetastarch, glycerol, polyvinylpyrrolidine (Rinfret, A. P., 1960, Ann. N.Y. Acad. Sci. 85:576), polyethylene glycol (Sloviter, H. A. and Ravdin, R. G., 1962, Nature 196:548), albumin, dextran, sucrose, ethylene glycol, i-erythritol, D-ribitol, D-mannitol (Rowe, A. W., et al., 1962, Fed. Proc. 21:157), D-sorbitol, i-inositol, D-lactose, choline chloride (Bender, M. A., et al., 1960, J. Appl. Physiol. 15:520), amino acids (Phan The Tran and Bender, M. A., 1960, Exp. Cell Res. 20:651), methanol, acetamide, glycerol monoacetate (Lovelock, J. E., 1954, Biochem. J. 56:265), and inorganic salts (Phan The Tran and Bender, M. A., 1960, Proc. Soc. Exp. Biol. Med. 104:388; Phan The Tran and Bender, M. A., 1961, in Radiobiology Proceedings of the Third Australian Conference on Radiobiology, Ilbery, P. L. T., ed., Butterworth, London, p. 59). Typically, the cells may be stored in 10% DMSO, 50% serum, and 40% RPMI 1640 medium. Methods of cell separation and purification are found in U.S. Patent No. 5,888,499, which is expressly incorporated by reference.

In a preferred embodiment, the PBMCs are then washed to remove serum proteins and soluble blood

components, such as autoantibodies, inhibitors, etc., using techniques well known in the art. Generally, this involves addition of physiological media or buffer, followed by centrifugation. This may be repeated as necessary. They can be resuspended in physiological media, preferably AIM-V serum free medium (Life Technologies) (since serum contains significant amounts of inhibitors of TGF-β) although buffers such as Hanks balanced salt solution (HBBS) or physiological buffered saline (PBS) can also be used.

Generally, the cells are then counted; in general from 1 X 10^9 to 2 X 10^9 white blood cells are collected from a 5-7 liter leukophoresis step. These cells are brought up roughly 200 mls of buffer or media.

In a preferred embodiment, the PBMCs may be enriched for one or more cell types. For example, the PBMCs may be enriched for CD8+ T cells, CD4+ T cells or stem cells. Cell types other than stem cells may be enriched for using commercially available immunoabsorbent columns, or using research procedures (the PBMCs are added to a nylon wool column and the eluted, nonadherent cells are treated with antibodies to CD4, CD16, CD11b, and CD74, followed by treatment with immunomagnetic beads, leaving a population enriched for CD8+ T cells). As more fully described below, isolation of stems cells, e.g., CD34+ stem cells, is done as is known in the art in a closed system using the Nexell separator.

In a preferred embodiment, cell populations are enriched for either CD8+ or CD4+ cells. In other embodiments, other cell types may be enriched for, such as CD3+CD4-CD8- or NK cells.

An advantage of using PBMCs is that other cell types within the PBMC population produce TGF-β, thus decreasing or even eliminating the requirement of the suppressive-inducing composition comprising TGF-β. Thus, in some embodiments, no enrichment step is used.

In a preferred embodiment, the CD4+ cells may be further purified to include only undifferentiated, naive cells. This is done by depleting them of CD45RO+ cells using monoclonal antibodies. This procedure eliminates populations of CD4+ cells which may have acquired functions which might interfere with the generation or activity of suppressor T cells.

Once the cells have undergone any necessary pretreatment, the cells are treated with a suppressive-inducing composition. By "treated" in this context herein is meant that the cells are incubated with the suppressive-inducing composition for a time period sufficient to result in T cell tolerance, particularly when transplanted into the recipient patient. The incubation will generally be under physiological temperature.

A suppressive-inducing composition includes at least one compound which induces T cells to become

tolerant to a recipient's cells. By "suppressive-inducing composition" herein is meant a composition that can induce T cell tolerance. Generally, these compositions are cytokines. Suitable suppressive-inducing compositions include, but are not limited to, IL-10, Il-2, Il-4, Il-15 and TGF-β.

Compounds used to induce T cell tolerance include, but are not limited to, prostalandins, nitric oxide, chemokines, cytokines and T cell activators. In a preferred embodiment, the compound used to induce tolerance is a cytokine.

A suppressive-inducing composition containing more than one compound may be used to induce T cells to become tolerant. The suppressive-inducing composition may contain more than one compound from the same class of compounds, i.e., two or more cytokines, chemokines, or prostaglandins may be mixed together. A suppressive-inducing composition also may contain compounds from different classes of compounds, such as a cytokine and a chemokine, or a cytokine and a prostaglandin, etc.

In a preferred embodiment, the suppressive-inducing composition comprises a mixture of cytokines, including TGF-β.

The concentration of the suppressive-inducing composition will vary depending on the identity of the composition, but will generally be at physiologic concentration, i.e. the concentration required to give the desired effect, i.e. an enhancement of specific types of regulatory cells. In a preferred embodiment, TFG-β is used in the suppressive-inducing composition. By "transforming growth factor-β" or "TGF-β" herein is meant any one of the family of the TGF-βs, including the three isoforms TGF-β1, TGF-β2, and TGF-β3; see Massague, (1980), *J. Ann. Rev. Cell Biol 6*:597. Lymphocytes and monocytes produce the ß1 isoform of this cytokine (Kehrl *et al.* (1991), *Int J Cell Cloning 9*:438-450). The TFG-β can be any form of TFG-β that is active on the mammalian cells being treated.

In humans, recombinant TFG-β is currently preferred. A human TGF-β2 can be purchased from Genzyme Pharmaceuticals, Farmington, MA. In general, the concentration of TGF-β used ranges from about 2 picograms/ml of cell suspension to about 2 nanograms, with from about 10 pg to about 500 pg being preferred, and from about 50 pg to about 150 pg being especially preferred, and 100 pg being ideal. In general, for immunosuppressive effects, about 1 ng/ml appears optimal. For the generation of suppressor cells it appears lower concentrations (e.g. about 0.1 ng/ml) are optimal.

In some embodiments, IL-2 is used as the suppressive-inducing composition. The IL-2 can be any form of IL-2 that is active on the mammalian cells being treated. In humans, recombinant IL-2 is currently preferred. Recombinant human IL-2 can be purchased from Cetus, Emeryville, CA. In general, the concentration of IL-2 used ranges from about 1 Unit/ml of cell suspension to about 100

U/ml, with from about 5 U/ml to about 25 U/ml being preferred, and with 10 U/ml being especially preferred. In a preferred embodiment, IL-2 is not used alone.

In a preferred embodiment, the invention provides methods for treating T cells with a suppressive-inducing composition and T cell activators, to generate regulatory T cells. A population of T cells which develop the capacity to prevent other T cells from proliferating and killing the recipient's cells, are referred to herein as "regulatory cells" or "suppressor cells".

By "T cell activator" herein is meant a compound which stimulates the expression of cytokine receptors. Suitable T cell activators include anti-CD3 and anti-CD28 antibodies, anti-CD2 antibodies combinations of monoclonal antibodies, specific autoantigens if known, cells from a transplant recipient (i.e. alloantigens), IL-2, IL-4, IL-15 and mitogens, such as Concanavalin A or staphylococcus enterotoxin B (SEB).

In a preferred embodiment cells are treated with a suppressive-inducing composition containing cytokine and a T cell activator. For example, cells from a transplant recipient may be used as the T cell activator in combination with a suppressive-inducing composition comprising TGF-β. Similarly, T cell activators such as anti-CD3 and anti-CD28 antibodies, anti-CD2 antibodies, Concanavalin A (Con A), or staphylococcus enterotoxin B (SEB) may be used with TGF-β. Other combinations of suppressive-inducing compositions and T cell activators are also possible, such as using the CD2 ligand, LFA-3 and cytokines such as IL-2, IL-4, and TNF-α.

When a mitogen, such as Concanavalin A or staphylococcus enterotoxin B (SEB) is used to stimulate the expression of cytokine receptors, it is generally used as is known in the art, at concentrations ranging from 1 μg/ml to about 10 μg/ml. In addition, it may be desirable to wash the cells with components to remove the mitogen, such as α-methyl mannoside, as is known in the art.

In a preferred embodiment, CD2 activators, such as a anti CD2 antibodies, which may include the CD2 ligand LFA-3, are used as activating agents. CD2 is a cell surface glycoprotein expressed by T lymphocytes. By "CD2 activator" herein is meant compound that will initiate the CD2 signaling pathway. A preferred CD2 activator comprises anti CD2 antibodies (OKT11, American Type Culture Collection, Rockville MD and GT2, Huets, et al., (1986) J. Immunol. 137:1420). In general, the concentration of CD2 activator used will be sufficient to induce the production of TGF-β. The concentration of anti CD2 antibodies used ranges from about 1 ng/ml to about 10 μg/ml, with from about 10 ng/ml to about 100 ng/ml being especially preferred.

In a preferred embodiment, cells from the transplant recipient are used to activate the donor CD4+ or CD8+ T cells. TGF-β is used in the suppressive-inducing composition which may also include other

cytokines such as IL-2, IL-4, and IL-15 to facilitate the effects of TGF-β.

The suppressive-inducing composition is incubated with the donor cells and a population of irradiated PMBC recipient cells (harvested as outlined above). The recipient cells are irradiated so that they cannot attack the donor cells, but will stimulate the donor cells to become tolerant to the recipient cells. The incubation occurs for a period of time sufficient to cause an effect, generally from 4 hours to 5 days although both shorter and longer time periods are possible.

Thus, the recipient cells function as an antigen-specific T cell activator. This activation is necessary for the generation of regulatory cells. Without being bound by theory, it appears that the recipient cells upregulate cytokine receptors on the donor cells, thereby enabling the donor cells to become responsive to TGF-β and develop into regulatory cells.

In a preferred embodiment, CD4+ T cells are treated with a suppressive-inducing composition comprising TGF-β and a T cell activator to develop contact-dependent suppression. These regulatory T cells are extremely potent and exert their suppressive effects even when they comprise < 1% of the total T cells.

In a preferred embodiment, regulatory CD4+ T cells may be expanded without effecting the suppressive properties of the cells. Preferably, CD4+ T cell may be expanded at least once. More preferably, CD4+ T cells may be expanded up to fourteen fold. More preferably, CD4+ cells may be expanded even further.

In a preferred embodiment, CD4+ T cells activated in the presence of TGF-β and repeatedly stimulated to produce immunosuppressive levels of active TGF-β. By "immunosuppressive levels of active TGF-β" herein is meant a concentration of TGF-β that has a direct immunosuppressive effect on cell function, that is, a concentration of TGF-β that renders the donor cells non responsive to a recipient's histocompatibility antigens. In general, concentrations of TGF-β > 500 pg/ml have immunosuppressive effects on cell function.

In one embodiment, treatment of the donor cells with the suppressive-inducing composition is followed by immediate transplantation into the recipient patient, generally after the cells have been washed to remove the suppressive-inducing composition. In this embodiment, after the donor cells have been conditioned or treated with the suppressive-inducing composition, they may be frozen or otherwise stored.

In a preferred embodiment, a second step is done comprising obtaining a population of donor hematopoietic stem cells from aspirated bone marrow or PBMCs. Stem cells comprise a very small

percentage of the white blood cells in blood, and are isolated as is known in the art, for example as described in U.S. Patent Nos. 5,635,387 and 4,865,204, both of which are incorporated by reference in their entirety, or harvested using commercial systems such as those sold by Nexell. As outlined above, CD34+ stem cells can be concentrated using affinity columns; the eluted cells are a mixture of CD34+ stem cells and lymphocytes. The contaminating lymphocytes are generally be removed using known techniques such as staining with monoclonal antibodies and removal using conventional negative selection procedures.

Once the CD34+ stem cells have been isolated, they may be mixed with the donor cells previously treated with the suppressive-inducing composition and immediately introduced into the recipient patient.

In one embodiment, the cells are treated for a period of time, washed to remove the suppressive-inducing composition, and may additionally reincubated. The cells are preferably washed as outlined herein to remove the suppressive-inducing composition. Further incubations for testing or evaluation may also be done, ranging in time from a few hours to several days. If evaluation of any cellular characteristics prior to introduction to a patient is desirable, the cells may be incubated for several days to several weeks to expand numbers of suppressor cells.

Once the cells have been treated, they may be evaluated or tested prior to transplantation into the patient. For example, a sample may be removed to do: sterility testing; gram staining, microbiological studies; LAL studies; mycoplasma studies; flow cytometry to identify cell types; functional studies, etc. Similarly, these and other lymphocyte studies may be done both before and after treatment. A preferred analysis is a test using labeled recipient cells; incubating the treated tolerant donor cells with a labeled population of the recipient cells will verify that the donor cells are tolerant and won't kill the recipient cells.

In a preferred embodiment, the treatment results in the conditioning of the T cells to become non-responsive to histocompatibility antigens of the recipient so that GVHD is prevented.

In a preferred embodiment, prior to transplantation, the amount of total or active TGF-β can also be tested. As noted herein, TGF-β is made as a latent precursor that is activated post-translationally.

After the cell treatment, the donor cells are transplanted into the recipient patient. The MHC class I and class II profiles of both the donor and the recipient are determined. Preferably, a non-related donor is found that preferably matches the recipients HLA antigens, but may mismatch at one or more loci if a matched donor cannot be identified. The recipient patient has generally undergone bone marrow ablation, such as a high dose chemotherapy treatment, with or without total body irradiation.

The donor cells are transplanted into the recipient patient. This is generally done as is known in the art, and usually comprises injecting or introducing the treated cells into the patient as will be appreciated by those in the art. This may be done via intravascular administration, including intravenous or intraarterial administration, intraperitoneal administration, etc. For example, the cells may be placed in a Fenwall infusion bag by injection using sterile syringes or other sterile transfer mechanisms. The cells can then be immediately infused via IV administration over a period of time, such as 15 minutes, into a free flow IV line into the patient. In some embodiments, additional reagents such as buffers or salts may be added as well.

After reintroducing the cells into the patient, the effect of the treatment may be evaluated, if desired, as is generally outlined above and known in the art.

The treatment may be repeated as needed or required. After a period of time, the leukemic cells may reappear. Because the donor lymphocytes are now tolerant to the recipient's cells, the patient now receives a transfusion of unconditioned donor lymphocytes which recognize the leukemic cells as foreign and kill these cells.

In a preferred embodiment, the invention further provides kits for the practice of the methods of the invention, *i.e.*, the incubation of the cells with the suppressive-inducing compositions. The kit may have a number of components. The kit comprises a cell treatment container that is adapted to receive cells from a donor. The container should be sterile. In some embodiments, the cell treatment container is used for collection of the cells, for example it is adaptable to be hooked up to a leukophoresis machine using an inlet port. In other embodiments, a separate cell collection container may be used.

The form and composition of the cell treatment container may vary, as will be appreciated by those in the art. Generally, the container may be in a number of different forms, including a flexible bag, similar to an IV bag, or a rigid container similar to a cell culture vessel. It may be configured to allow stirring. Generally, the composition of the container will be any suitable, biologically inert material, such as glass or plastic, including polypropylene, polyethylene, etc. The cell treatment container may have one or more inlet or outlet ports, for the introduction or removal of cells, reagents, suppressive-inducing compositions, etc. For example, the container may comprise a sampling port for the removal of a fraction of the cells for analysis prior to introduction into the recipient patient. Similarly, the container may comprise an exit port to allow introduction of the cells into the recipient patient; for example, the container may comprise an adapter for attachment to an IV setup.

The kit further comprises at least one dose of a suppressive-inducing composition. "Dose" in this context means an amount of the suppressive-inducing composition such as cytokines, that is sufficient

to cause an effect. In some cases, multiple doses may be included. In one embodiment, the dose may be added to the cell treatment container using a port; alternatively, in a preferred embodiment, the dose is already present in the cell treatment container. In a preferred embodiment, the dose is in a lyophilized form for stability, that can be reconstituted using the cell media, or other reagents.

In some embodiments, the kit may additionally comprise at least one reagent, including buffers, salts, media, proteins, drugs, etc. For example, mitogens can be included.

In some embodiments, the kit may additional comprise written instructions for using the kits.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All patents, patent applications, publications and references cited herein are expressly incorporated by reference.

EXAMPLES

Example 1

Treatment of donor CD8+ Cells ex vivo to suppress an immune attack against blood cells of

A blood sample from a donor was obtained and lymphocytes prepared by density gradient centrifugation. T cells were prepared using a conventional negative selection procedure. These T cells were conditioned to prevent them from attacking the recipient cells. For this conditioning, the CD8+ T cells were mixed with irradiated stimulator cells from the recipient. The stimulator cells were derived from T cell-depleted blood cells from the recipient. The mixture of donor T cells and recipient stimulator cells were cultured for 48 hours with different concentrations of one or more cytokines. In this example the cytokines were TGF-β and IL-10. This procedure abolished the potential of the donor T cells to kill recipient cells, in Figure 4B.

To test the ability of the donor T cells to recognize and kill recipient blood cells, the donor T cells were cultured with irradiated stimulator cells for 5 days. Then the donor cells were cultured for 4 hours with a sample of recipient radiolabeled blood cells. When the recipient's cells are killed they release radioisotope into the culture medium. By determining the amount of radioisotope released, one can calculate the percentage of cells killed. In the standard cytotoxicity assay shown in Figure 4A, donor cells were cultured with labeled recipient cells in 30 to 1, 15 to 1, and 7.5 to 1 ratios. These combinations of donor and recipient cells are called effector to target ratios. Killing is indicated by the symbols. As expected, maximum killing was seen at the highest effector to target cell ratio. In Figure

4A, the open circles shows that if 30 donor cells were mixed with 1 recipient cell, 40 percent of the recipient cells were killed. When donor T cells were conditioned with very small concentrations of TGF-β (0.01 or 0.1 nanograms per ml), they had no effect on killing (Figure 4D). However, if the T cells were treated with 1 nanogram per ml of TGF-β, the killing of recipient cells decreased by 50 percent. Figure 4B shows that if the T cells were treated with IL-10, killing also decreased by 50%. If the T cells had been conditioned with both IL-10 and TGF-β at 1 nanogram per ml, these cells completed blocked the killing of recipient cells; killing was almost undetectable. Various combinations of mitogens, cytokines, and monoclonal antibodies can be used to make T cells non-responsive.

Example 2

Treatment of donor CD8+ T cells *ex vivo* to prevent other donor T cells from mounting an immune attack against blood cells from an unrelated individual (i.e. inhibiting GVHD).

CD8+ cells can be conditioned by TGF-β to suppress the cytotoxic immune response of one individual against the cells of an unrelated donor. In this manner, one can assess the cytotoxic activity of donor A against donor B's histocompatibility mismatched blood mononuclear cells. To develop suppressor cells, purified CD8+ cells from donor A were cultured with irradiated T cell-depleted mononuclear cells from donor B with TGF-β added. Separately, the cytotoxic activity of donor A's T cells against donor B cells was documented. Figure 5 shows that the addition of TGF-β conditioned CD8+ T Cells from donor A significantly decreased the resulting cytotoxic activity. The addition of control CD8+ cells had a minimal effect. In these experiments, CD8+ T cells from donor A were activated by foreign antigens displayed by donor B cells. Additional IL-2 was not needed. These experiments reveal that TGF-β not only induces CD8+ T cells to suppress antibody production, but also induces CD8+ cells to suppress cell-mediated immune responses.

Example 3

Treating a patient with chronic myelocytic leukemia with the stem cells from a histoincompatible donor: tolerization with mitogens

The harvested PBMC of the donor are placed in a sterile container in HBBS as in Example 1. The cells are then incubated with mitogens to induce lymphocytes to become non-responsive to histocompatibility antigens of the recipient. In this case the cells are incubated with physiological concentrations of concanavalin A (Con A) for 4 to 72 hours using standard incubation techniques. The concentration of Con A used can range from about 0.01 to about 10 micrograms/ml with 1 microgram/ml being presently preferred. Alternatively, SEB may be used as the mitogen at concentrations of 0.001 to 100 ng/ml.

The incubation of the mononuclear cells in the mitogen solution increases the population of T

suppressor cells. These cells, when transferred to the recipient, will enable the stem cells to engraft without causing GVHD. Although it is not known how the mitogens work, it is believed to induce the production of TGF-beta by certain mononuclear cells in preparation, and the TGF-beta acts on T cells to become suppressor cells ro to directly redner other T cells non responsive.

After the cells have been incubated with the mitogens, the cells are washed with HBBS to remove any mitogens that are in the solution. The cells are suspended in 200-500 ml of HBBS, mixed with the stem cells and administered to a patient with CML who has been treated with myeloablative agents to prepare the stem cells for engraftment.

Once the donor hematopoietic cells lymphocites engraft in the recipient, and the patient again becomes healthy and free of leukemic cells. If the leukemic cells recur, the patient receives a transfusion of donor lymphocites and the leukemic cells again disappear.

Example 4

Treating a patient with chronic myelocytic leukemia with the stem cells from a histoincompatible donor: use of anti-CD2 monoclonal antibodies to induce production of TGF-β.

The harvested enriched stem cell preparation of the donor are placed in a sterile container in HBBS as in Example 1. The cells are then incubated with anti-CD2 monoclonal antibodies to induce lymphocytes to become non-responsive to histocompatibility antigens of the recipient. In this case, the cells are incubated with anti-CD2 monoclonal antibodies for 4 to 72 hours using standard incubation techniques. The concentration of anti-CD2 monoclonal antibodies are 10 ng/ml to 10 ug/ml. Optionally, 1-1000 units of IL-2 can be added.

The incubation of the mononuclear cells in the anti-CD2 solution increases the population of T suppressor cells. These cells, when transferred to the recipient will enable the stem cells to engraft without causing GVHD. It is believed that incubation with anti-CD2 monoclonal antibodies induces the production of TGF-beta by certain monuclear cells in preparation, and the TGF-beta acts on T cells to become suppressor cells.

After the cells have been incubated with the anti-CD2 monoclonal antibodies, the cells are washed with HBBS to remove antibodies that are in the solution. The cells are suspended in 200-500 ml of HBBS mixed with the stem cells harvested previously and administered to a patient with CML who has been treated with myeloblative agents to prepare the stem cells for engraftment.

Once the donor hematopoietic cells lymphocytes engraft in the recipient, and the patient again

becomes healthy and free of leukemic cells. If the leukemic cells recur, the patient receives a transfusion of donor lymphocytes and the leukemic cells again disappear.

Example 5

Treating a patient with chronic myelocytic leukemia with the stem cells from a histoincompatible donor: tolerization with mitogens and cytokines.

The harvested PBMC of the donor are placed in a sterile container HBBS as in Example 1. The cells are then incubated with cytokines and mitogens to induce lymphocytes to become non-responsive to histocompatibility antigens of the recipient. In this case the cells are incubated with physiological concentrations of Con A or SEB, TGF-beta, and/or IL-2, IL-4 or IL-15 and for 4 to 72 hours using standard incubation techniques.

After the cells have been incubated with the cytokines and mitogens, the cells are washed with HBBS to remove any cytokine and mitogen that are in the solution. The cells are suspended in 200-500 ml of HBBS mixed with the stem cells and administered to a patient with CML who has been treated with myeloabative agents to prepare the stem cells for engraftment.

Once the donor hematopoietic cells and lymphocytes engraft in recipient and the patient again becomes healthy and free of leukemic cells. If the leukemic cells recur, the patient receives a transfusion of donor lymphocytes and the leukemic cells again disappear.

Example 6

Treating a patient with chronic myelocytic leukemia with the stem cells from a histoincompatible donor; tolerization with a mitogen and cytokine.

The harvested PBMC of the donor are placed in a sterile container in HBBS as in Example 1. The cells are then incubated with a cytokine and a mitogen to induce lymphocytes to become non-responsive to histocompatibility antigens of the recipient. In this case the cells are incubated with physiological concentrations of ConA, and IL-2 for 4 to 72 hours using standard incubation techniques. In another case, SEB could be used.

After the cells have been incubated with the cytokines and mitogens, the cells are washed with HBBS to remove any cytokine and mitogen that are in the solution. The cells are suspended in 200-500 ml of HBBS mixed with stem cells and administered to a patient with CML who has been treated with myeloablative agents to prepare the stem cells for engraftment.

Example 7

Effects of TGF-β on purified CD8+ Cells

Treatment of CD8+ with TGF-β effects CD8+ cells in at least three ways. First, treatment of T cells with PMA (20 ng/ml) and ionomycin (5 μM) in the presence TGF-β upregulates the expression of CD40 Ligand (Figure 1). Secondly, IL-2 expression is in enhanced in T cells stimulated with ConA in the presence of TGF-β (Figure 3). Thirdly, TGF-β increases TNF-α expression by CD8+ cells when purified CD8+ cells are stimulated for 24 hours with Con A (5 μg/ml) ± TGF-β (10 pg/ml) ± IL-2 (10 U (Figure 2).

Example 7

Treatment of CD4+ cells *ex vivo* to prevent an immune attack

Instead of using mitogens to induce regulatory T cells, the allogeneic mixed lymphocyte reaction is used for this purpose. In this reaction, T cells from donor A recognize and respond to foreign histocompatibility antigens displayed by PBMCs from donor B. These responder T cells proliferate and develop the capacity to kill donor B's cells.

To develop suppressor T cells, various CD4+ cell subsets from donor A were cultured with irradiated T cell-depleted mononuclear cells from donor B. The cells were cultured for 5 days with or without TGF-β (1ng/ml) in the suspensions. After this time, TGF-β was removed and the cells added to fresh T cells from donor A and non-T cells from donor B.

Naive CD4+ cells developed potent suppressive activity when activated in the presence of TGF-β. These CD4+ T cells had the capacity to block the generation of CTL activity against allogeneic target cells. In a two step co-culture experiment, CD4+ CD45RA+ RO- T cells were first incubated with irradiated allogeneic stimulator cells ± TGF-β1 for 5 days. Various numbers of these cells were then added to fresh T cells and stimulator cells and the effect of these cells on the generation of CTL activity was assessed. Whereas control CD4+ cells had minimal to moderate suppressive activity, those that had been primed in the presence of TGF-β almost completely blocked responder T cells from killing allogeneic target cells in a standard 4 hour chromium release assay (Figure 6A). Activation of CD4+ cells was needed for the development of inhibitory activity. T cells cultured with TGF-β without stimulation do not develop this suppressive effect (20). CD4+ suppressor cells primed with alloantigens in the presence of TGF-β are referred to herein as "CD4reg". Naive CD8+ T cells primed with stimulator cells also develop moderate suppressive activity, but unlike CD4+ cells, the presence of TGF-β did not enhance this activity (Figure 6B).

Figure 7 shows two additional experiments with CD4+ regulatory T cells induced by TGF-β.

Further studies revealed that regulatory CD4+ T cells generated in this manner have a unique mode of action. Unlike the CD8+ and CD4+ T cells generated previously which suppress by secreting inhibitory cytokines, these allo-specific regulatory CD4+ T cells have a contact dependent mechanism of action (Figure 8).

Activation of naive CD4+ T cells in the presence of TGF-β enabled them to respond more vigorously to alloantigens, accelerated their conversion to the activated phenotype, and increased their viability. Figure 9 indicates properties of control and TGF-β conditioned CD4+ cells after 5 days of culture. Allo-stimulated CD4+ cells displayed the typical features of activation and some were already undergoing activation-induced cell death as indicated by Annexin V staining. Importantly, those alloactivated in the presence of TGF-β were even larger, a fraction stained more intensely with CD4, and expression of CD25 was markedly increased. In 13 experiments mean values for CD25 expression increased from undetectable levels in naive cells to 42.3 ± 3.6% of CD4+ cells alloactivated in the presence of TGF-β. Mean values for control alloactivated CD4+ cells were 34.5 ± 3.8%, ($p<0.01$%, paired *t* test). Intracellular and surface expression of CTLA-4 was increased in CD4reg compared with control CD4+ cells (intracellular: 31.5 ± 4.1% vs. 23.2 ± 4.5%, $p< 0.01$; surface: 10.8 ± 0.9% vs. 5.2 ± 0.4%, $p< 0.01$). A greater percentage of CD4reg had downregulated surface expression of CD45RA. Significantly, annexin V staining was markedly decreased and the total number of CD4reg recovered was 56% greater than control CD4+ cells. Thus, although CD4reg were more intensively activated that control CD4 cells, they were also more resistant to activation-induced apoptosis.

Similar to the murine CD4+ regulatory T cells described by Shevach and co-workers (Suri-Payer, E., et al.l, *J. Immunol.* 160:1212-1218, 1998; Suri-Payer, E., et al., *Eur. J. Immunol.* 29:669-677, 1999; Thornton, A.M., and Shevach, E.M., *J. Exp. Med.* 188:287-296, 1998), separation of CD4reg into CD25+ and CD25- fractions by cell sorting revealed that almost all of the suppressive activity was contained in the CD25+ fraction (Figure 10A). The CD4+ CD25- fraction displayed only minimal suppressive activity. Moreover, the CD4+ CD25+ were very potent as indicated by their ability to markedly inhibit the generation of CTL activity in very low numbers. Figure 10B shows that suppressive activity was only slightly diminished when the numbers of the CD4+ suppressor cells was reduced from 25 to 3 percent of total T cells. With this small number of added CD4+ cells the minimal suppressive activity mediated by the CD25- subset had disappeared.

Unlike CD4+ regulatory T cells generated by repeated stimulation with IL-10, which have low proliferative capacity (Groux, H., et al., *Nature.* 389:737-742. 24, 1997), CD4reg induced in the presence of TGF-β proliferated in response to IL-2 and retained their suppressive capacity. The CD25+ fraction of CD4reg was isolated by cell sorting and cultured for 5 days with IL-2. These cells expanded 7 to 14 fold during this time and retained their suppressive function in three separate

experiments. Figure 11 shows strong inhibition of the generation of CTL activity when these CD4reg comprised only 5% of the total T cells. Moreover, this suppressive activity remained when the number of these CD4+ CD25 cells was reduced to only 0.2% of total cells. Irradiation of these regulatory T cells, however, abolished their suppressive effects.

Addition of these T cells to responder T cells and allo-stimulator cells inhibited proliferation (Figure 12) and decreased the ability of responder CD8+ killer precursor cells to become activated (Figure 13).

CD4reg blocked the proliferative response of responder T cells to alloantigens (Figure 14B) and CD8+ T cells were the principal target. Gating on CD8+ cells by flow cytometry after a 5 day culture, alloactivated CD8+ cells displayed the expected marked increase in CD25, increased expression of CD69 and Fas, and evidence of cell division by CFSE-labeled and propidium iodide-labeled effector T cells. Moreover, some CD8+ cells were undergoing activation-induced cell death as indicated by annexin V staining. In sharp contrast, in cultures containing CD4reg, upregulation of CD25, CD69 and Fas by CD8+ cells was markedly inhibited and almost none underwent apoptosis or cell division. Studies of the absolute numbers of CD8+ cells in culture revealed only a minimal change from the total count. Thus, CD4reg rendered CD8+ cells anergic.

Example 9

CD4+ T cells can be stimulated to produce immunosuppressive levels of TGF-β

CD4+ T cells that produce immunosuppressive levels of TGF-β have been named Th3 cells, but the mechanisms involved in their development are poorly understood. We have obtained evidence that strong stimulation of CD4+ cells with the superantigen, staphylococcus enterotoxin B (SEB), or repeated stimulation of CD4+ cells stimulated with a lower concentration of SEB induced these cells to produce immunosuppressive levels of active TGF-β.

Figure 15 shows increased production of both active and total TGF-β produced by CD4+ T cells stimulated with increasing concentrations of SEB. Figure 16 shows the effect of repeated stimulation of CD4+ T cells with low doses of SEB. By the third time these T cells were stimulated with SEB, they produced significant amounts of the active form of TGF-β.

Figure 17 shows the effects of SEB on naive (CD45RA+ CD45RO-) CD4+ and CD8+ T cells. The cells were stimulated with SEB every 5th day for a total of three stimulations. The percentages of each T cell subset and the cells expressing the CD25 IL-2 receptor activation marker were determined after each stimulation. Panels A and C show that by including TGF-β 1ng/ml in the initial stimulation, CD4+ T cells became the predominant subset in the cultures after repeated stimulation. Panels B and D show that CD25 expression by SEB stimulated cells decreased by the third stimulation in control

cultures. However, CD25 expression remained very high if the T cells were primed with TGF-β. Thus, TGF-β appears to have preferential effects on CD4+ cells if these T cells are repeatedly stimulated and almost all of these cells were CD25+ after culture for 20 days.

In summary, following T cell stimulation, the initial regulatory effects of TGF-β are directed to CD8+ cells. Upon more prolonged or repeated stimulation, this cytokine now induces CD4+ cells to become regulatory cells and these cells are more potent than CD8+ cells in their suppressive activities.

Example 10

Treating a patient with chronic myelocytic leukemia
who has developed GVHD following the stem cell transplant.

In the instance that the initial procedure to prevent early or late GVHD following the stem cell transplant is not successful, this event will be managed by transfer of a larger number of donor T cells that have been conditioned to become suppressor cells. Approximately 1 x 10^9 PBMCs obtained by leukopheresis are concentrated in a sterile leukopak; in Hanks balanced salt solution (HBBS). The PMMCs will be separated into CD4+ and CD8+ cells by magnetic beads coated with the appropriate monoclonal antibodies. The cells will be treated with the desired T cell activating agent and suppressive-inducing composition for the optimum time to develop maximal regulatory activity. The cells will be expanded and then transferred to the recipient. These conditioned T cells migrate to lymphoid organs and suppress the GVHD.

Besides chronic myelocytic leukemia, other hematologic malignancies such as acute and chronic leukemias, lymphomas, solid tumors such as breast carcinoma or renal cell carcinoma among a few , and non-malignant diseases such as severe anemias (thalassemia, sickle cell anemia) can be treated with mismatched allogeneic stem cells.

Another aspect of this invention is a kit to perform the cell incubation with the cytokines. The kit comprises a sterile incubating container with the appropriate concentration of cytokines preloaded within the container. In one embodiment of the kit, the cytokines are present in lyophilized form in the container. The container is preferably a bag, similar to an IV bag. The lyophilized cytokines are reconstituted with HBBS and then the cells are injected into the container and thoroughly mixed and incubated. In another embodiment of the invention the cytokines are already in solution within the container and all that has to be done is the injection of washed stem cell preparation and incubation.

CLAIMS

What is claimed is:

1. A method for inducing T cell tolerance in a sample of *ex vivo* peripheral blood mononuclear cells (PBMCs) comprising adding a suppressive-inducing composition to said cells.

2. A method for treating donor cells to ameliorate graft versus host disease in a recipient patient comprising:

a) removing peripheral blood mononuclear cells (PBMC) from a donor;

b) treating said cells with a suppressive-inducing composition for a time sufficient to induce T cell tolerance; and

c) introducing said cells to said patient.

3. A method according to claim 1 or 2 wherein said suppressive-inducing composition comprises TGF-β and IL-2.

4. A method according to claim 1 or 2 further comprising treating said donor cells with a T cell activator.

5. A method according to claim 4 wherein said T cell activator is a recipient cell.

6. A method according to claim 2 wherein said method further comprises adding said cells to donor stem cells prior to introduction into said patient.

7. A method according to claim 1 or 2 wherein said PBMCs are enriched for CD8+ cells.

8. A method according to claim 1 or 2 wherein said PBMCs are enriched for CD4+ cells.

9. A method for generating suppressor cells in a sample of ex vivo peripheral blood mononuclear cells (PBMCs) comprising adding a suppressive-inducing composition to said cells.

10. A method for treating donor cells to ameliorate graft versus host disease in a recipient patient comprising:

a) removing peripheral blood mononuclear cells (PBMC) from a donor;

b) treating said cells with a suppressive-inducing composition for a time sufficient to generate suppressor cells; and

c) introducing said cells to said patient.

11. A method according to claim 9 or 10 wherein said suppressive-inducing composition comprises TGF-β.

12. A method according to claim 9 or 20 wherein said suppressive-inducing composition comprises a mixture of IL-2 and TGF-β.

13. A method according to claim 9 or 10 further comprising treating said donor cells with a T cell activator.

14. A method according to claim13 wherein said T cell activator is a recipient cell.

15. A method according to claim 10 wherein said method further comprises adding said cells to donor stem cells prior to introduction into said patient.

16. A method according to claim 9 or 10 wherein said PBMCs are enriched for CD8+ cells.

17. A method according to claim 9 or 10 wherein said PBMCs are enriched for CD4+ cells.

18. A kit for the treatment of donor cells comprising:

a) a cell treatment container adapted to receive cells from a donor; and

b) at least one dose of a suppressive-inducing composition.

19. A kit for the treatment of donor cells according to claim 18 further comprising at least one dose of a T cell activator.

20. A kit according to claim 18 or 19 further comprising written instructions for the method of treating.

21. A kit according to claim 18 or 19 wherein said dose is contained within said cell treatment container.

22. A kit according to claim 18 or 19 wherein said dose is in a lyophilized form.

23. A kit according to claim 18 or 19 wherein said cell treatment container further comprises at least one reagent.

24. A kit according to claim 18 or 19 wherein said cell treatment container further comprises a sampling port to enable the removal of a fraction of said cells for analysis.

25. A kit according to claim 18 or 19 further comprising an exit port adapted to enable transport at least a portion of said cells to a recipient patient.

26. A kit according to claim 18 wherein said suppressive-inducing composition is a mixture of IL-2 and TGF-β.

27. A kit according to claim 19 wherein said T cell activator is a mitogen.

28. A kit according to claim 27 wherein said mitogen is staphylococcal enterotoxin B.

ABSTRACT

The field of the invention is generally related to pharmaceutical agents useful in treating graft-versus-host disease (GVHD) in patients that have received allogenic bone marrow transplants.

1028454

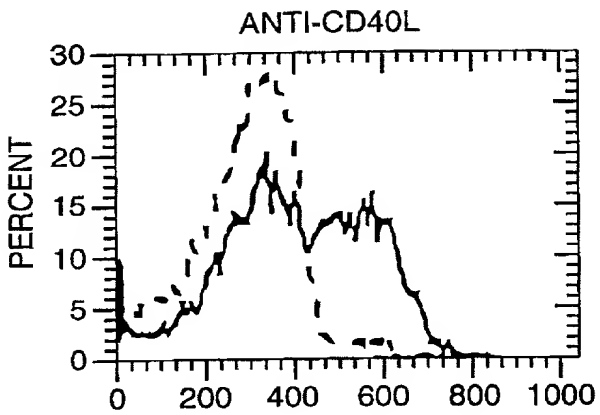

FIG._1A

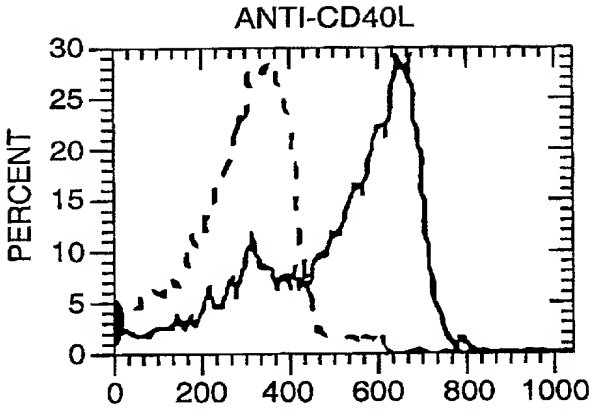

FIG._1B

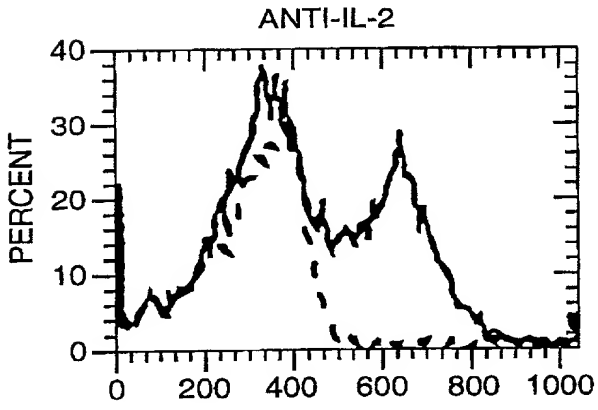

FIG._3A

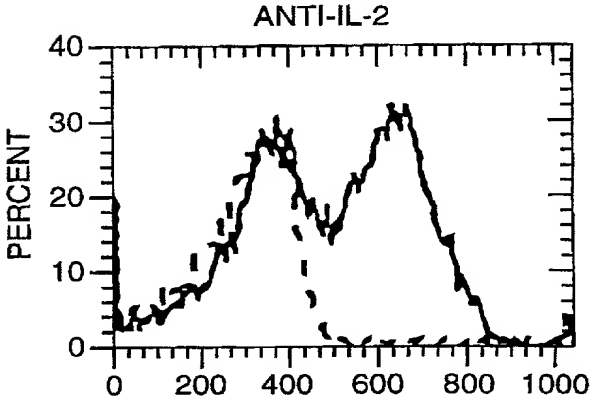

FIG._3B

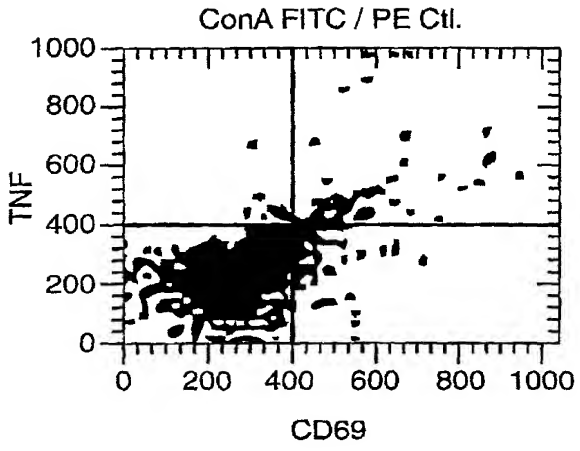

FIG._2A

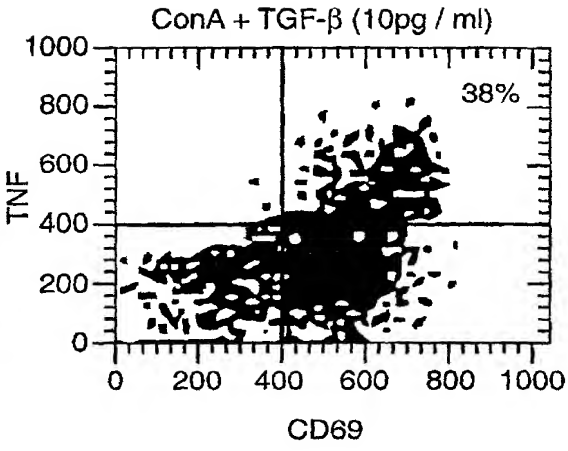

FIG._2B

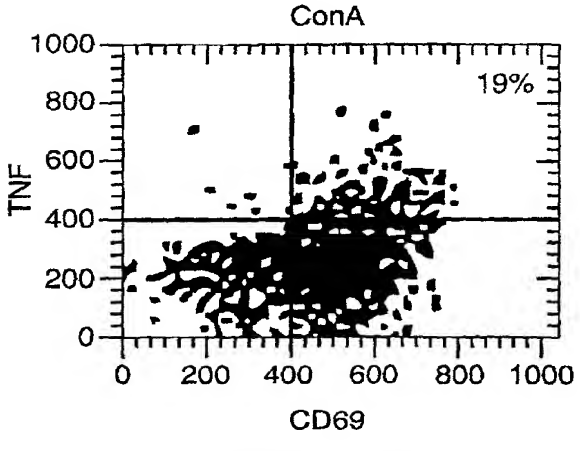

FIG._2C

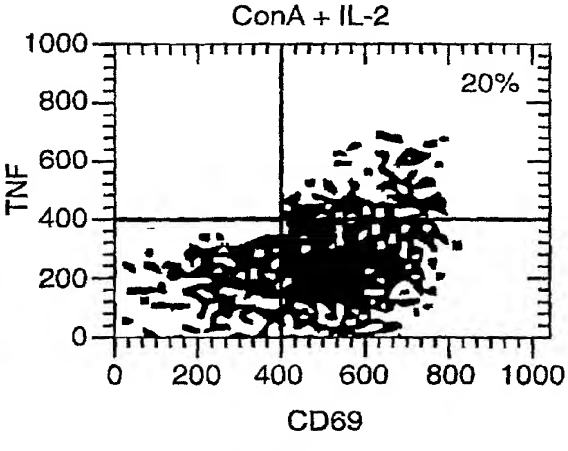

FIG._2D

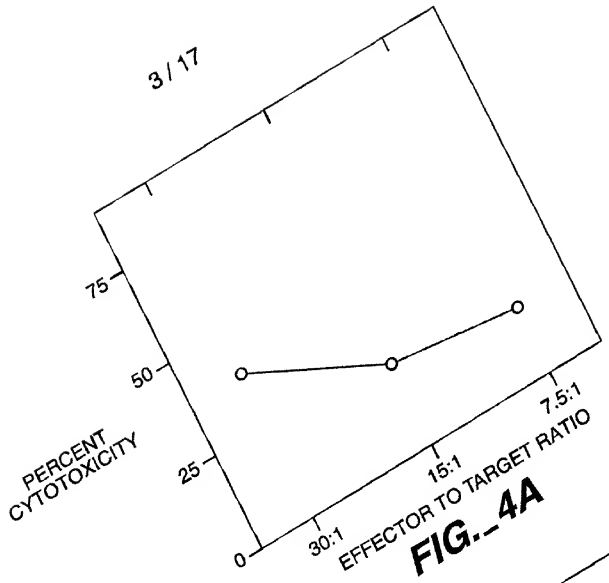
75
50
25
0
PERCENT CYTOTOXICITY
30:1
15:1
7.5:1
EFFECTOR TO TARGET RATIO

FIG._4A

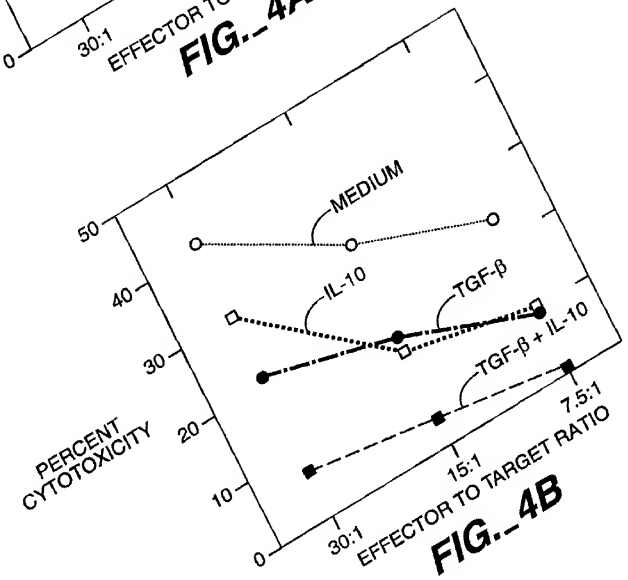
50
40
30
20
10
0
PERCENT CYTOTOXICITY
MEDIUM
IL-10
TGF-β
TGF-β + IL-10
30:1
15:1
7.5:1
EFFECTOR TO TARGET RATIO

FIG._4B

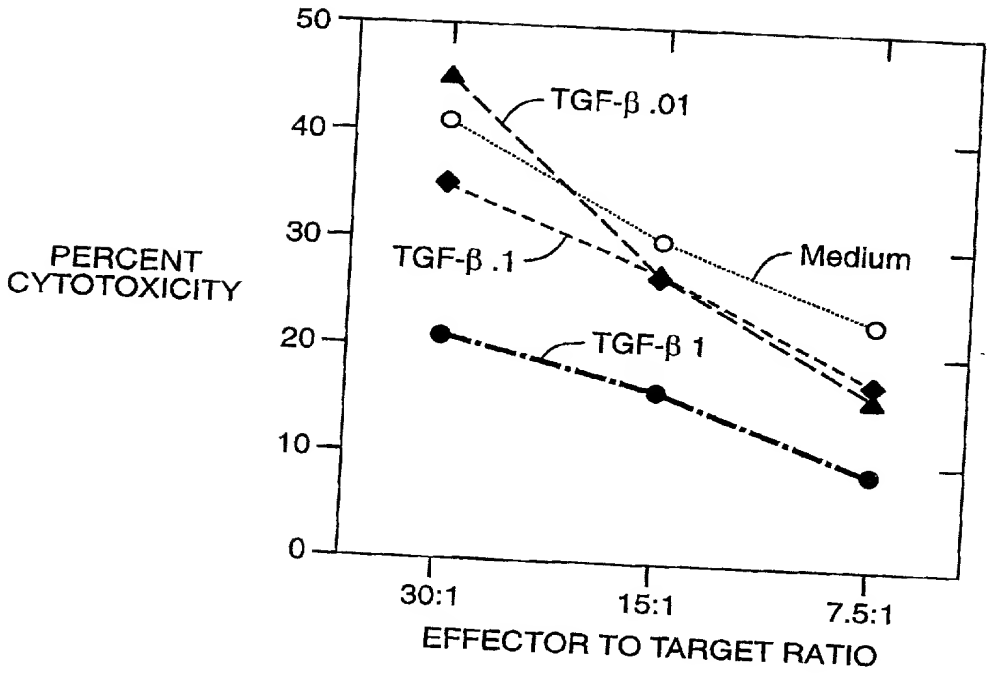

FIG._4C

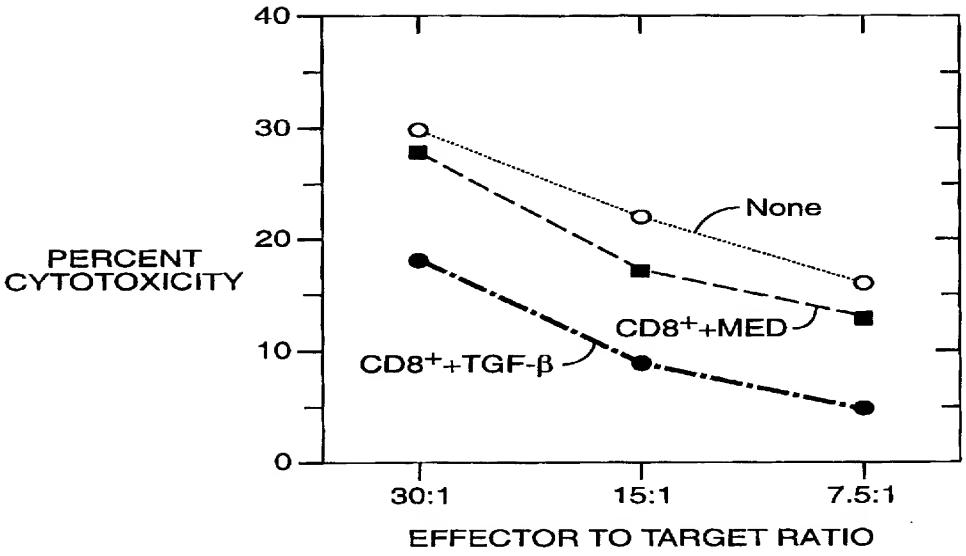
40
30
20
10
0
PERCENT
CYTOTOXICITY
None
CD8+MED
CD8+TGF-β
30:1
15:1
7.5:1
EFFECTOR TO TARGET RATIO

FIG._5

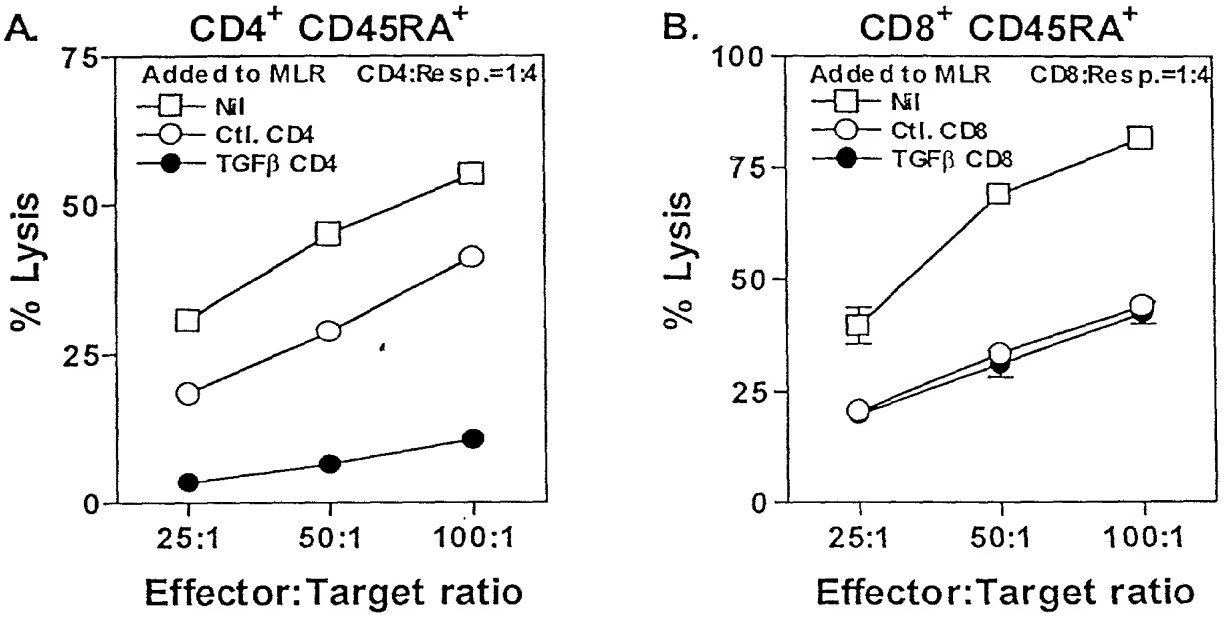
A.
CD4+ CD45RA+
Added to MLR CD4:Resp.=1:4
Nil
Ctl. CD4
TGFβ CD4
% Lysis
75
50
25
0
25:1
50:1
100:1
Effector:Target ratio
B.
CD8+ CD45RA+
Added to MLR CD8:Resp.=1:4
Nil
Ctl. CD8
TGFβ CD8
% Lysis
100
75
50
25
0
25:1
50:1
100:1
Effector:Target ratio

FIGURE 6

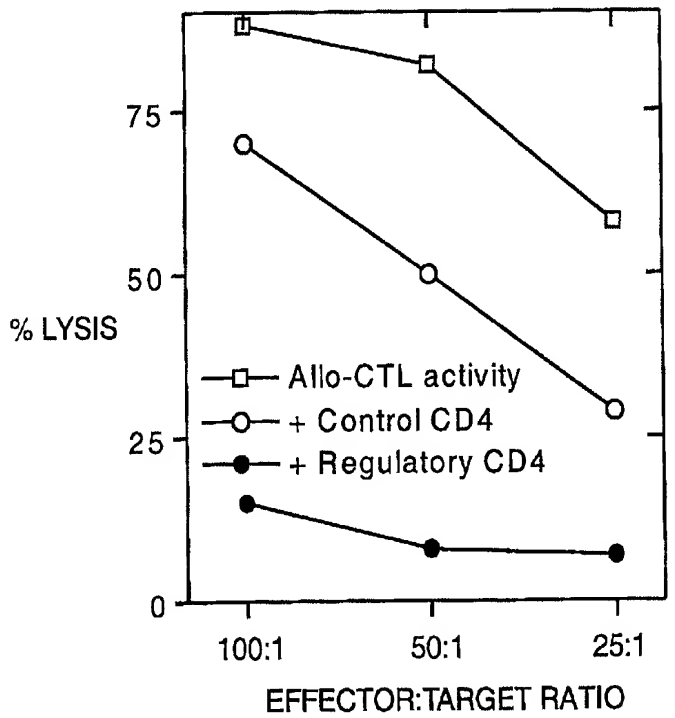
75
50
25
0
% LYSIS
Allo-CTL activity
+ Control CD4
+ Regulatory CD4
100:1
50:1
25:1
EFFECTOR:TARGET RATIO

FIG._7A

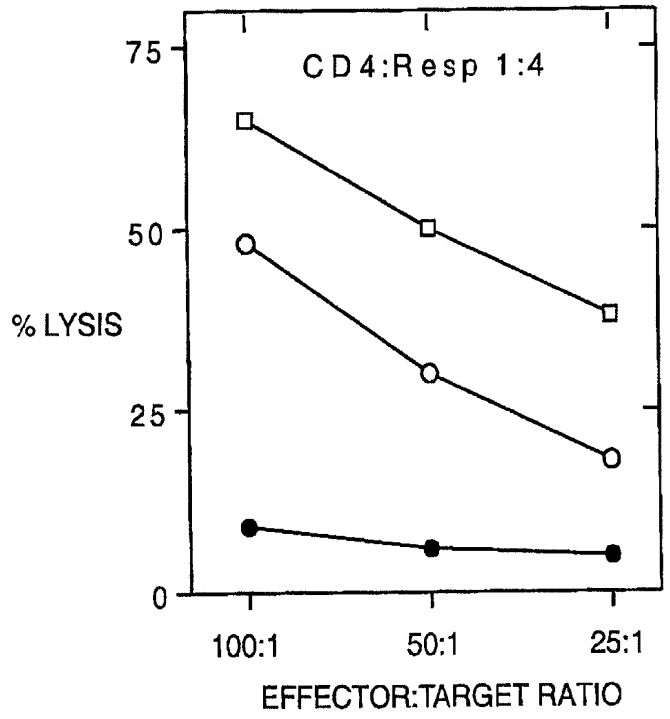
CD4:Resp 1:4
75
50
25
0
% LYSIS
100:1
50:1
25:1
EFFECTOR:TARGET RATIO

FIG._7B

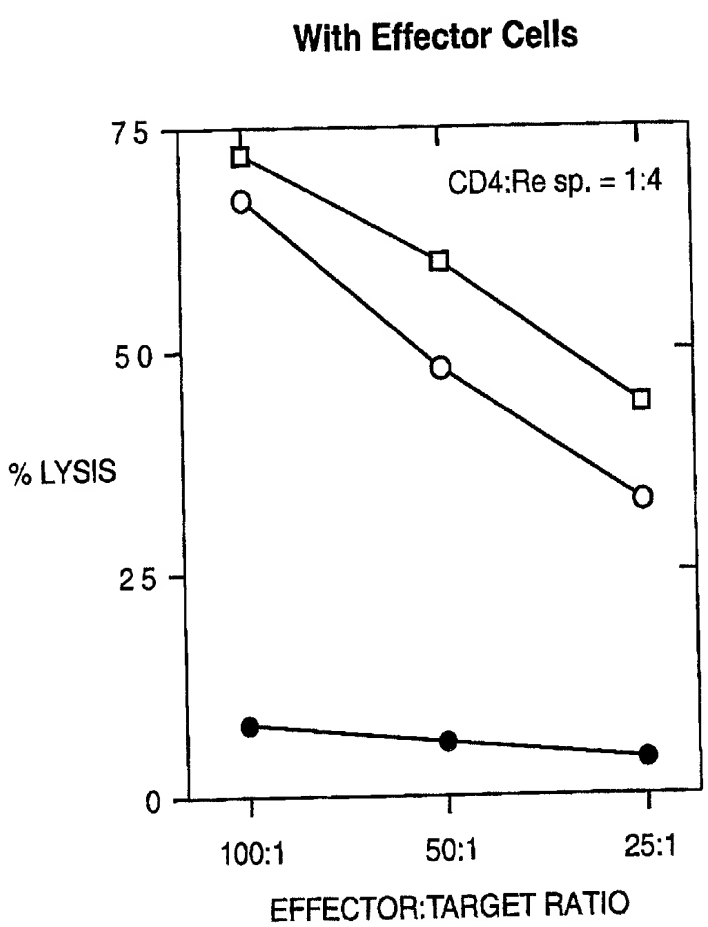
With Effector Cells
75
50
25
0
% LYSIS
CD4:Re sp. = 1:4
100:1
50:1
25:1
EFFECTOR:TARGET RATIO

FIG._8A

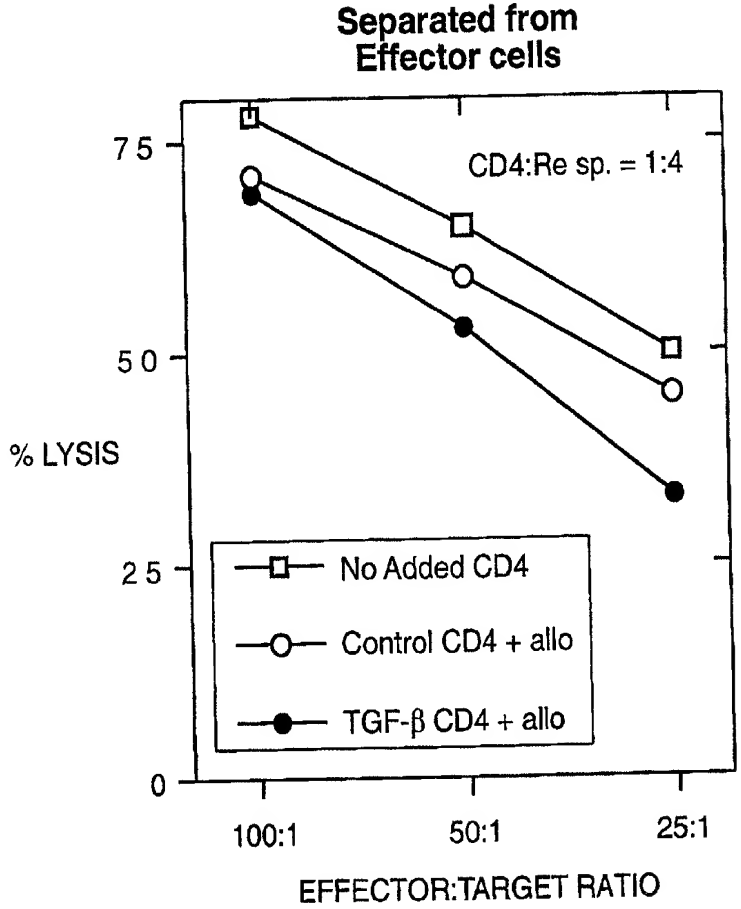
Separated from Effector cells
75
50
25
0
% LYSIS
CD4:Re sp. = 1:4
No Added CD4
Control CD4 + allo
TGF-β CD4 + allo
100:1
50:1
25:1
EFFECTOR:TARGET RATIO

FIG._8B

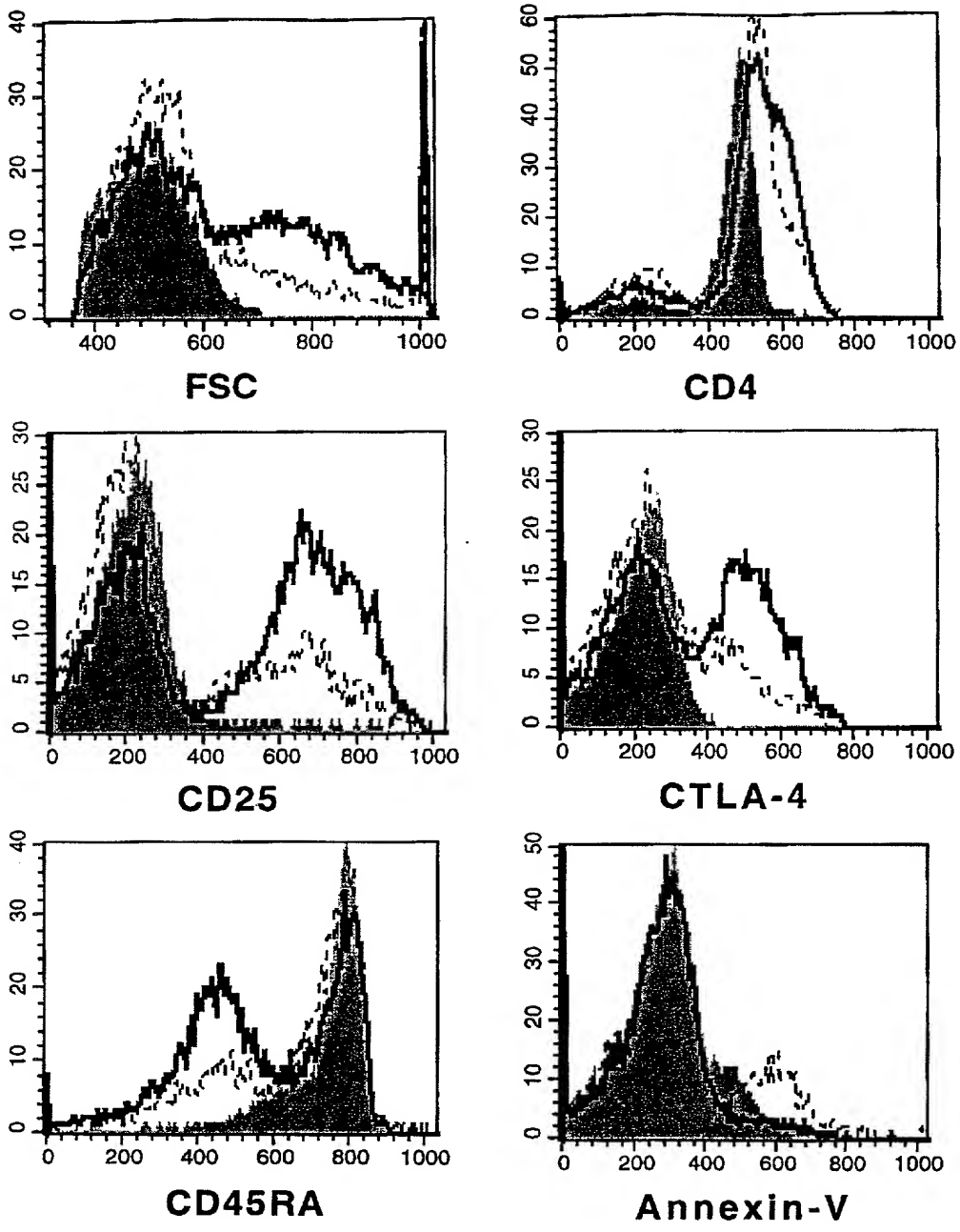

FIG._9

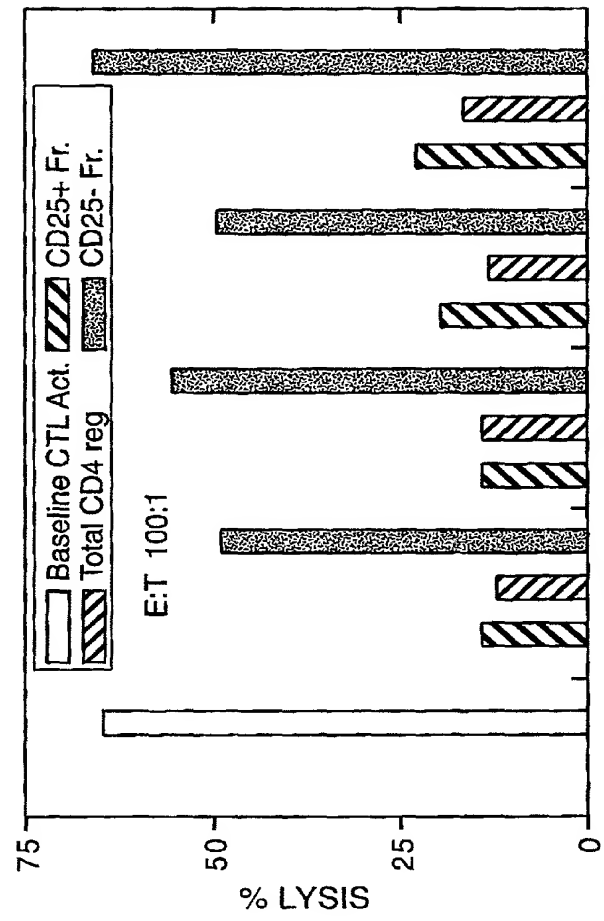

FIG._10B

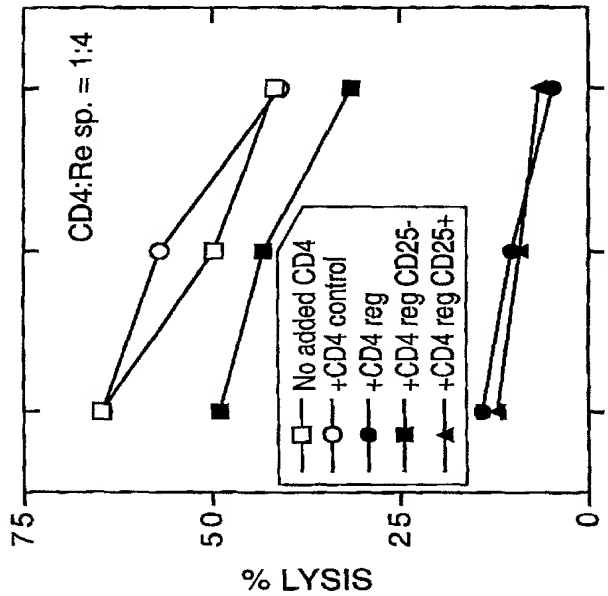

FIG._10A

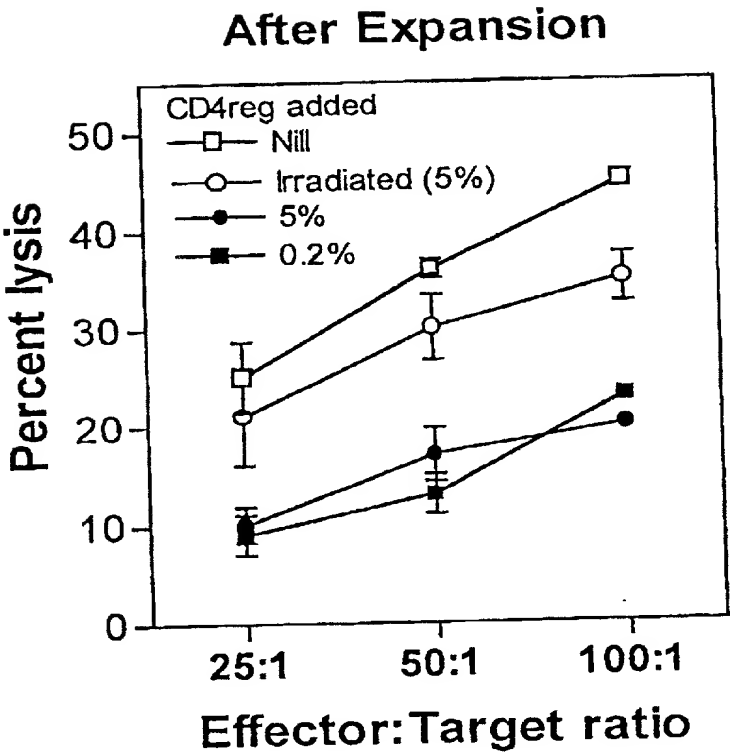
After Expansion
CD4reg added
Nill
Irradiated (5%)
5%
0.2%
Percent lysis
50
40
30
20
10
0
25:1
50:1
100:1
Effector:Target ratio

FIG._11

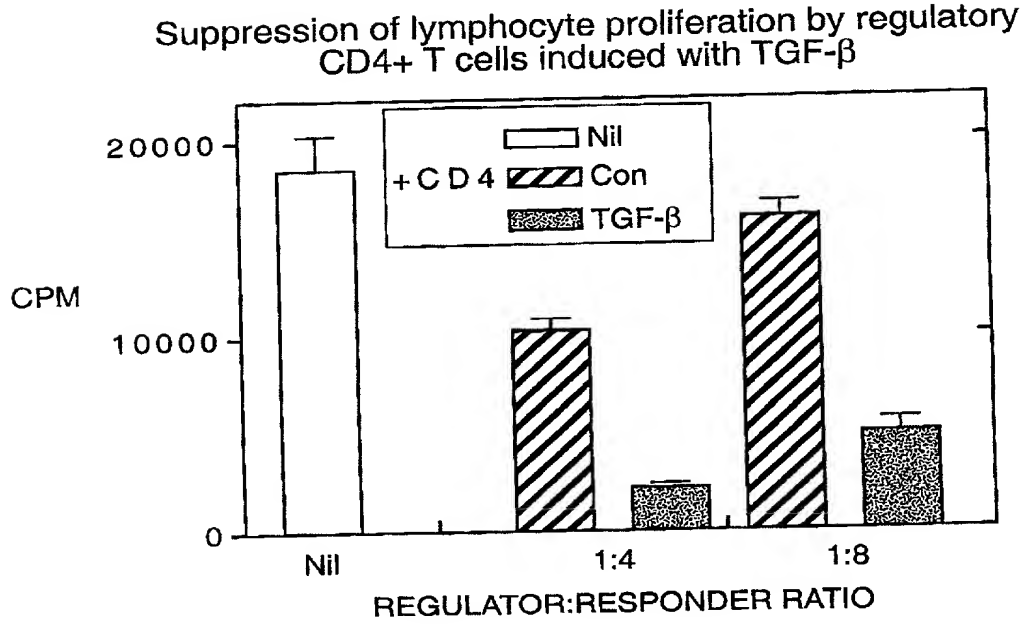
Suppression of lymphocyte proliferation by regulatory CD4+ T cells induced with TGF-β
Nil
+CD4 Con
TGF-β
CPM
20000
10000
0
Nil
1:4
1:8
REGULATOR:RESPONDER RATIO

FIG._12

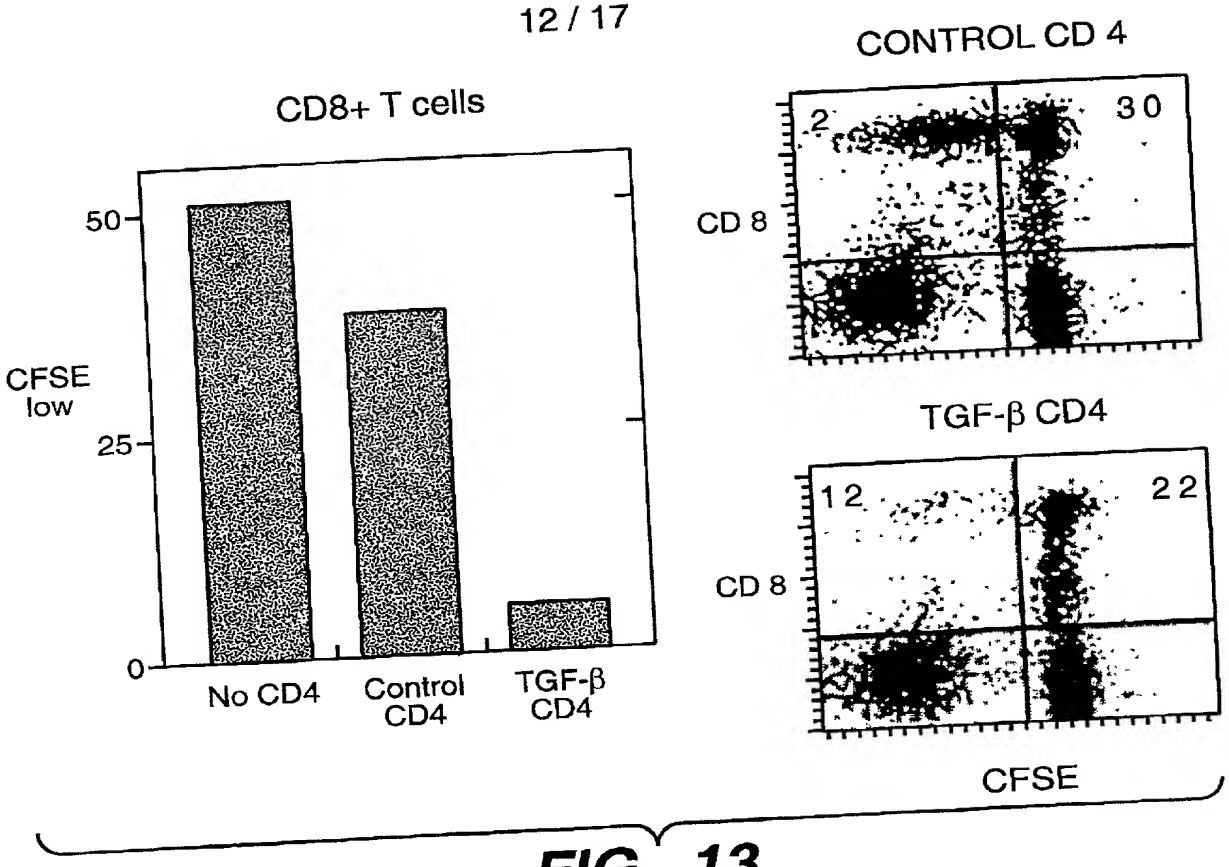

FIG._13

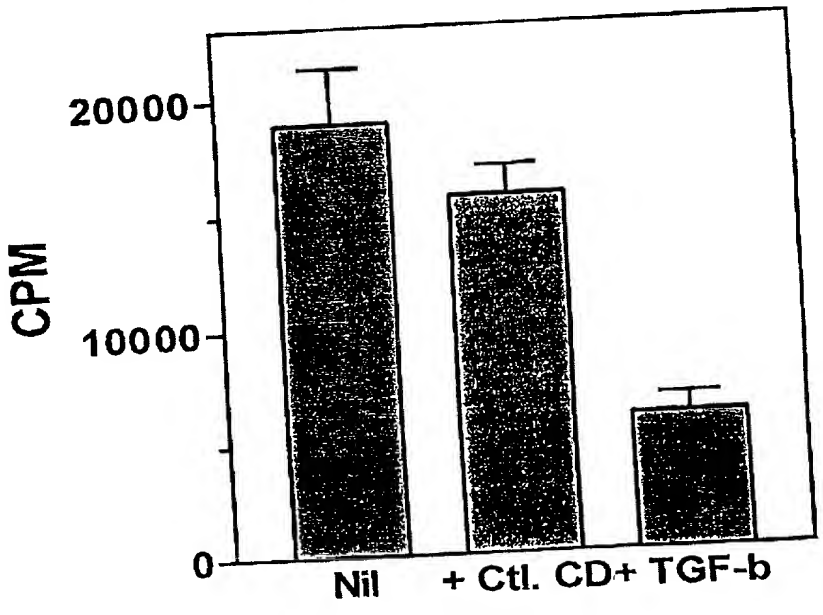

FIG._14A

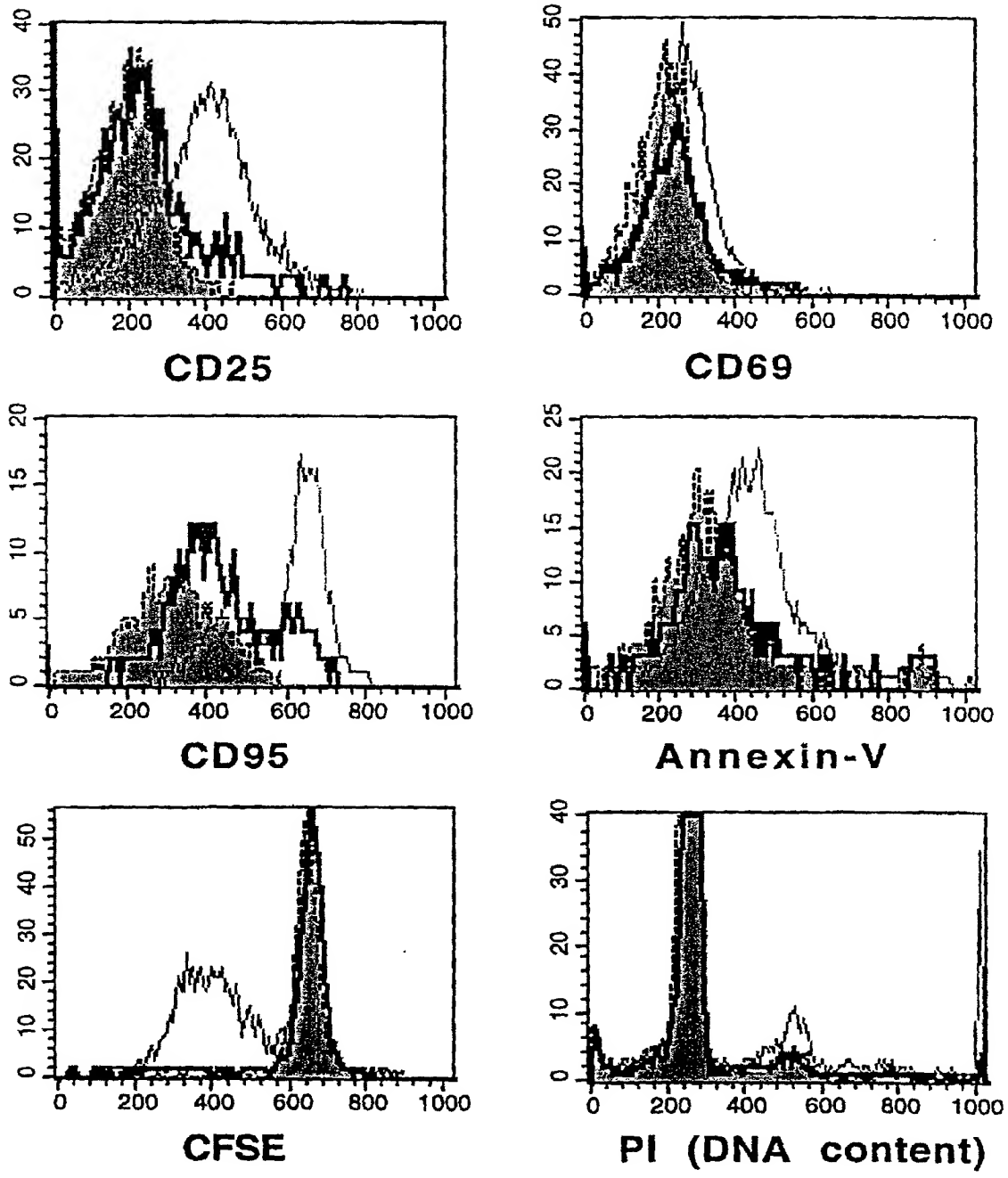

FIG._14B

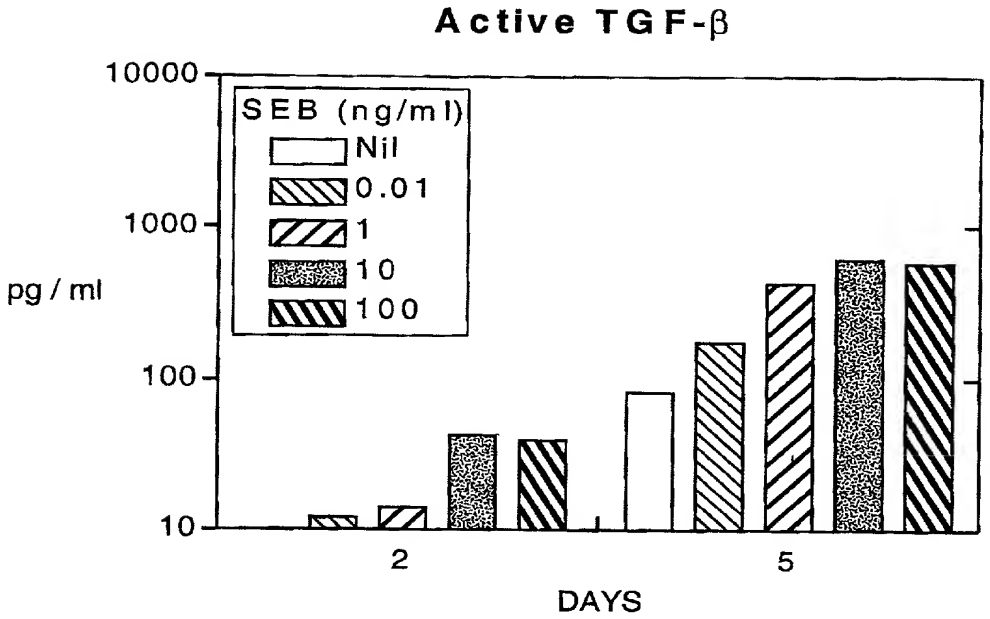

FIG._15A

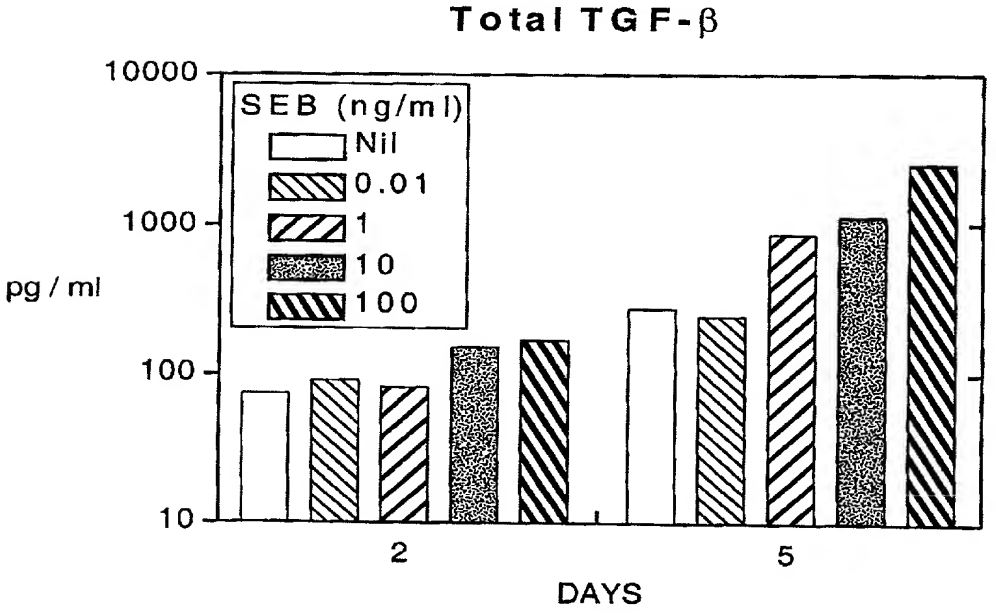

FIG._15B

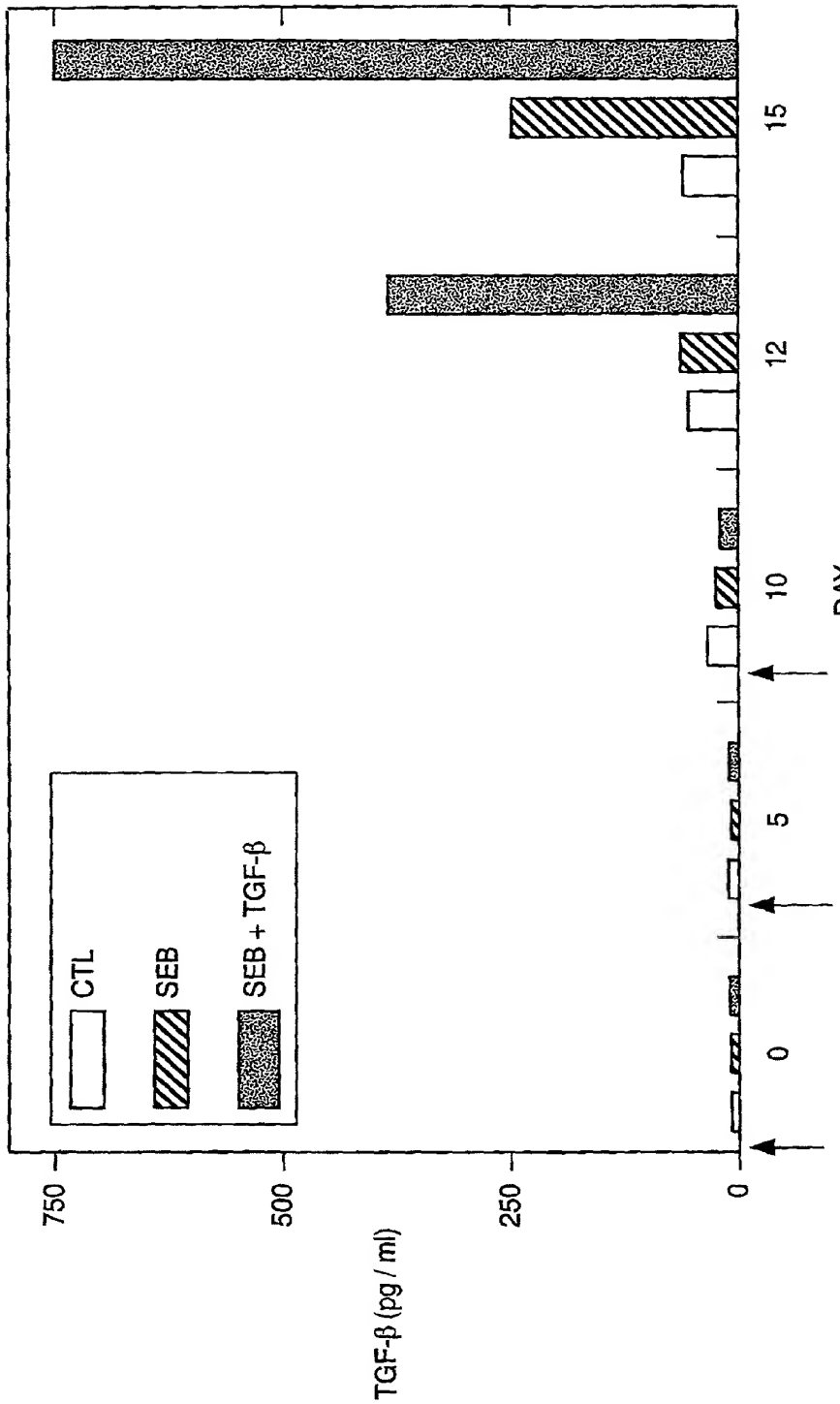

FIG._16

Untreated

T cell subset

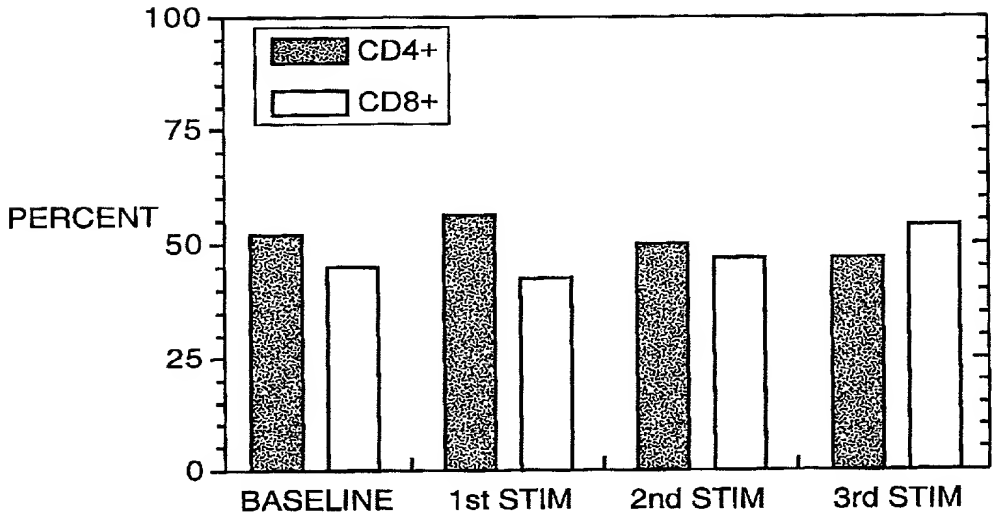

FIG._17A

Untreated

CD25 Expression

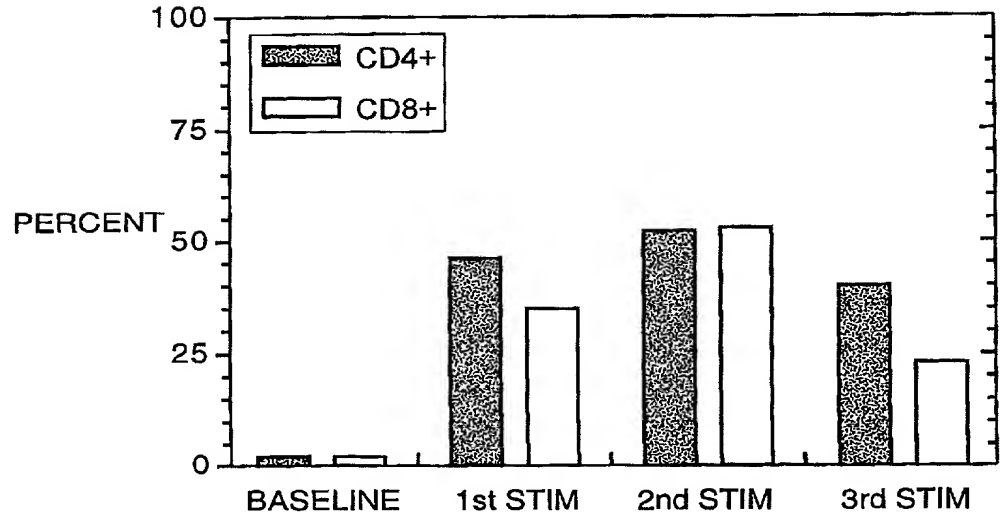

FIG._17B

TGF-β Treated

T cell subset

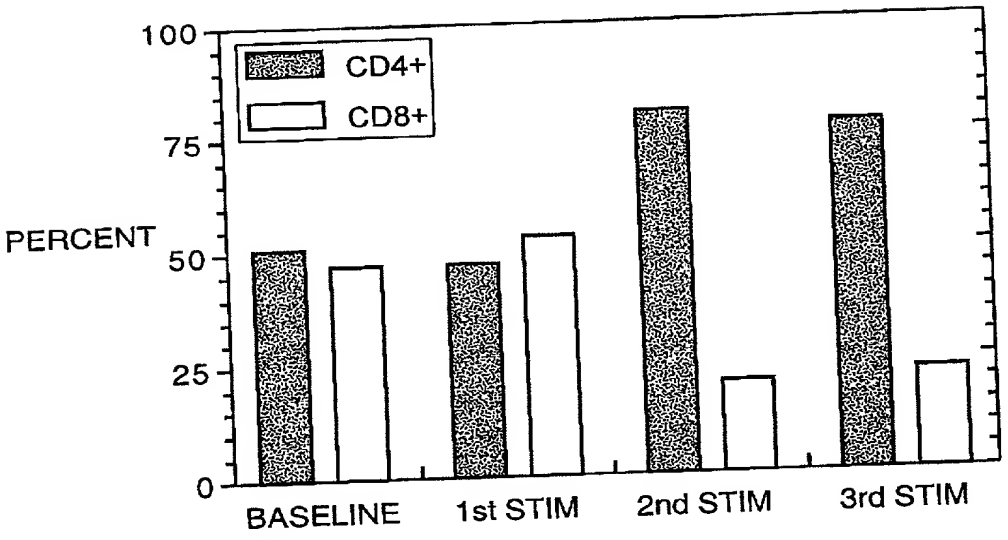

FIG._17C

TGF-β Treated

CD25 Expression

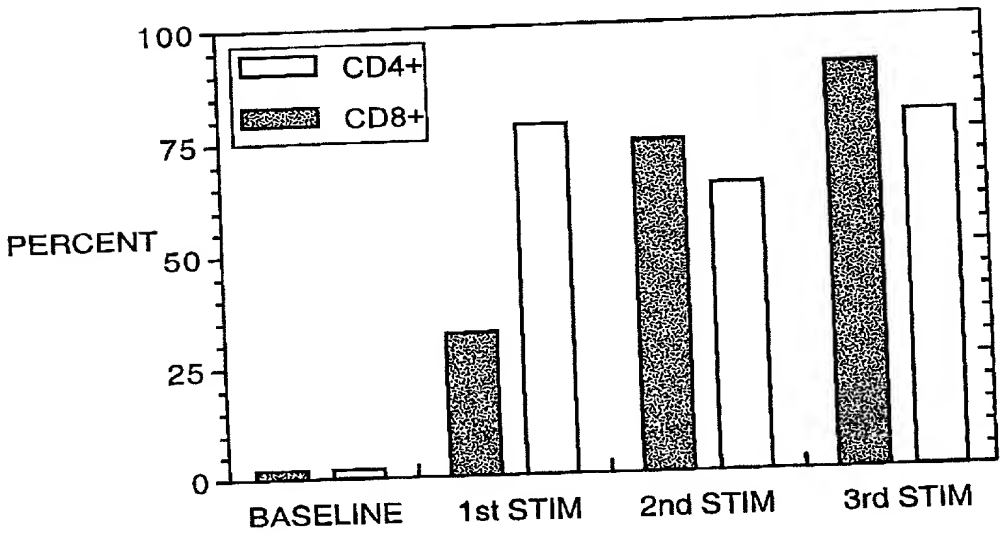

FIG._17D